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Immunomodulatory Effects of Surgical Trauma and Blood Transfusion

By

AHMAD GHAREHBAGHIAN

A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of the Doctoral of Philosophy in the Faculty of Medicine.

Department of Transplantation Sciences

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Summary

Major surgical trauma and allogeneic transfusions cause immunomodulation, a systemic condition characterised by a reduction in natural killer (NK) cell function, macrophage migration, antigen presentation, pro-inflammatory cytokine synthesis and lymphocyte blastogenesis. Clinically there is increased tumour recurrence, increased post-operative infections, delayed wound healing, prolonged hospital stay and increased mortality. Immunomodulation affects innate immunity as reflected in NK cell function. Hence a method was developed to measure the NK cell precursor frequency (NKpf) in samples of peripheral blood mononuclear cells (PBMC). The technique was based on the principle of limiting dilution analysis and was performed in the presence of recombinant interleukin two (rIL-2) and rIL-15. After five day's culture lysis of the K562 cell-line was measured and NKpf calculated. NKpf was measured before and after joint replacement surgery in 120 orthopaedic patients assigned to five groups according to the type of transfused blood they received, namely: allogeneic non-leukodepleted; allogeneic leukodepleted; autologous unwashed blood salvaged post-operatively from the operation site (autologous salvaged), and autologous pre-deposit. The fifth group was non-transfused. Interferon-gamma (IFNγ), IL-10 and IL-4 synthesis were measured in supernatants of similar cultures and flow-cytometry was used to measure percentage of the CD3 negative cells that were CD56 positive in the five-day cultures.

The results showed all groups had significantly decreased post-operative NKpf ($p<0.05$), and non significant decreased post-operative IFNγ and IL-10 synthesis ($p>0.05$) except the autologous salvaged group. By contrast, the latter showed a significant rise in post-operative NKpf values ($p<0.05$) and a non significant increase in IFNγ and IL-10 synthesis ($p>0.05$). IL-4 and immunophenotyping studies were inconclusive. The proportion developing post-operative infections in those who received allogeneic blood (non-leukodepleted

and leukodepleted) was 15% compared to 8% in those who received autologous blood (pre-deposit and salvaged) and 12% in the non-transfused group.

It was concluded that, major surgical trauma could be associated with impairment of NK cell potential, decreased IFNγ and IL-10 synthesis and that allogeneic transfusions add to these effects even after leukodepletion. These effects could lead to an increased risk of post-operative infections. By contrast autologous salvaged blood reverses these systemic effects perhaps by transferring locally synthesised cytokines and chemokines from the operation site to the circulation.

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DEDICATION

I would like to dedicate my work to my wonderful wife Parinaz for her support, understanding, encouragement and hard work as wife and mother.

I would also like to dedicate my work to my precious kids, Arshia and Avishan. I wish the very best for them in their life.

DECLARATION

This thesis is the result of my own work and investigations, except those where indicated in the acknowledgement section. Views expressed in this dissertation are those of myself and not of the university.

Ahmad Gharehbaghian

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Abbreviations:

^{51}Cr: Chromium 51

Ab-Ag-Ab: Antibody-Antigen-Antibody

ADCC: Antibody dependent cell cytotoxicity

Ag-Ab: Antigen-Antibody

AIDS: Acquired immunodeficiency syndrome

APC: Antigen presenting cell

ARDS: Adult respiratory distress syndrome

c.p.s.: Count per second

CAM: Cellular adhesion molecule

CCM-1: Complete culture media-1

CCM-2: Complete culture media-2

CD: Cell differentiation

CJD: Cretutzfeldt-Jakob disease

CMV: Cytomegalovirus

Con-A: Concanavalin-A

COX-2: Cyclooxygenase-2

CsA: Cyclosporin A

CSF: Colony stimulating factor

CSIF: Cytokine synthesis inhibitory factor

DMSO: Dimethyl sulfoxide

DTH: Delayed type hypersensitivity

DTPA: Diethylene triamino pentacetate

EBV: Epstein-Barr virus

ECM: Extra-cellular matrix

ELISA: Enzyme-linked immunosorbent assay

Eu: Europium

Eu-DTPA: Europium-Diethylene triamino pentacetate

FasL: Fas ligand

PHA: Phytohaemagglutinin

FNHTRs: Febrile non-haemolytic transfusion reactions

G-CSF: granulocyte colony stimulating factor

GM-CSF: Granulocyte/macrophage colony stimulating factor

GVHD: Graft versus host disease

HBV: Hepatitis B virus

Hct: haematocrit

HCV: Hepatitis C virus

Hepatitis NANBNC: Hepatitis non-A non-B non-C

HIV: Human immunodeficiency virus

HLA: Human leukocyte antigen

HSV: Herpes simplex virus

HTLV I/II: Human T-cell leukaemia/lymphoma virus I/II

IFNγ: Interferon-gamma

IFNα/β: Interferon-alpha/beta

IFNγR: Interferon-gamma receptor

Ig: Immunoglobulin

Ig-like: Immunoglobulin-like

IL-1α/β: Interlukin-1α/β

IL-10: Interlukin-10

IL-15: Interlukin-15

IL-2: Interleukin-2

IL-2R: Interlukin-2 receptor

IL-4: Interlukin-4

IL-8: Interlukin-8

IP-10: Interferon-induced protein-10

ITAM: Immunoreceptor tyrosine-based activation motifs

ITIM: Immunoreceptor tyrosine-based inhibitory motifs

LAK cell: Lymphokine-activated killer cell

LCMW: Lymphocytic choriomeningitis virus

LDA: Limiting dilution analysis

K562: The HLA negative NK sensitive human erythro-leukaemic cell line

KIR: Killing inhibitory receptor

KL (c-kit): c-kit ligand

LFA-1: Leukocyte function antigen-associated 1

LPS: Lipopolysaccharide

M-CSF: Macrophage colony stimulating factor

MCP: Macrophage chemotactic protein

MHC: Major histocompatibility complex

MHSC: Multipotent haemopoietic stem cell

MIP: Macrophage inflammatory protein-1α

MIG: Monokine induced by interferon

MOF: Multiple organ failure

MQ/MO: Macrophage/Monocyte

NCR: Natural cytotoxicity receptors

NF-kB: Nuclear factor-kappa B

NK cell: Natural killer cell

NKp: Natural killer precursor

NKpf: Natural killer precursor frequency

NKT-cell: Natural killer T-cell

nvCJD: new-variant Cretutzfeldt-Jakob disease

PBMC: Peripheral blood nuclear cell

PBS: Phosphate buffer saline

PGE_2: Prostoglandin-E_2

PHA: Phytohemoagglutinin

PMN: Polymononuclear

RAG: Recombinase activity genes

RANTES: Regulated upon activation normal T-cell expressed and secreted

RBC: Red blood cell

ROM: Reactive oxygen metabolites

RT: Room temperature

SCF: Stem cell factor

SAGM: Saline, adenine, glucose and mannitol

SCID: Severe combined immunodeficiency

TcR: T-cell receptor

TGF-β: Transforming growth factor-beta

THR: Total hip replacement

TKR: Total knee replacement

TNFα: Tumour necrosis factor-alpha

TNFR: Tumour necrosis factor receptor

VLA: Very late antigen

WBC: White blood cell

Section 1.

Introduction

1.1. Innate immunity

The immune system provides an effective defence system to meet infectious micro-organisms through two distinct pathways, innate immunity and adaptive immunity (Klein and Horejsi, 1997; Janeway et al., 2001)

Innate immunity provides a fast recognition and response system to most pathogens. The anatomical components of innate immunity include skin and mucosal surfaces as the first major external barrier protecting the body from exposure to pathogens. The cellular components include polymorphonuclear (PMN) cells (neutrophils, eosinophils, and basophils), monocytes/macrophages, dendritic cells and natural killer (NK) cells. These cells are connected by a wide range of soluble mediators including defensins, cathelicidins, chemokines, cytokines and complement. Complement and other soluble mediators can directly bind to conserved molecular structures on pathogens leading to opsonisation by phagocytes and release of soluble mediators that act as chemo-attractants or activators of leukocytes and mast cells (Klein and Horejsi, 1997; Janeway et al., 2001)

Innate immunity curbs infection and neoplasia, and is essential to the development of primary adaptive immunity. This begins with soluble mediators activating dendritic cells and attracting lymphocytes and NK cells to the site of infection and inflammation. It also up-regulates major histocompatibility complex (MHC) Class II molecules expression by release of interferon gamma (IFNγ) thereby amplifying the antigen-presentation pathway that is essential to the development of adaptive immunity (Trinchieri and Perussia, 1984).

1.1.1. Natural Killer (NK) cells

Natural killer (NK) cells are bone marrow derived mononuclear cells that recognise and kill target cells without previous sensitisation. They are also an important source of cytokines such as IFNγ. In the 1970s, NK cells were discovered during studies of natural cell-mediated cytotoxicity to virus-induced tumours in rodents and some cancer patients (Nunn et al., 1976). This new cell population capable of lysing certain tumour cells in the absence of prior sensitisation was named natural killer cells due to their ability to kill tumour cell lines without previous stimulation (Nunn et al., 1976; Trinchieri, 1989; Reyburnetal.,1997).

Morphologically NK cells are large granular lymphocytes, with a kidney shaped nucleus and azurophilic cytoplasmic granules. In the resting state they have average diameters of 7-8 μm and in the activated state10-12 μm. NK cells are present in fetal liver and spleen as early as 6- 8 weeks of gestation. They make up 5 to 15% of the total lymphocytes circulating in the blood. They represent up to 50% of total resident lymphocyte population in liver and around 5-10% of splenic lymphocytes in adults and children. They constitute 2-7% of the lymphocyte population in the lymph node and 30% in the lung (Herberman, 1981; Hata et al., 1991; Whiteside and Herberman, 1994).

T-cells and NK cells are both derived from multipotent haemopoietic stem cells, but NK cells do not require recombinase activity genes (RAG) for development, whereas T-cells do (Lanier, 2000a). Maturation pathways of human NK cells are complex and poorly understood. They develop primarily, but not exclusively along non-thymic pathways. Long-term bone marrow cultures showed that CD3-CD56+ NK cells developed from the CD34+/HLA-DR-/Lin- bone marrow cells in the presence of rIL-3, rIL-2, macrophage inflammatory protein-1 alpha (MIP-1α) and stem cell factor (SCF is also called c-kit ligand, KL) in the presence of stromal cell line (Miller et al., 1998), the fms-like tyrosine kinase 3 ligand (flt3L), SCF and IL-7 (Punzel et al., 1999), IL-2, IL-3, IL-7, SCF and flt3L in the presence of the murine stromal cell line (Miller et al., 1999), IL-15 and SCF

(Colucci and Di_Santo, 2000), flt3L, IL-7, IL-3 and SCF(McKenna et al., 2000). These grown cells in long-term culture express the characteristic CD3-CD56+ phenotype of NK cells, but not the T-cell (CD3+ CD5+ TcR), monocyte (CD14+ CD15+), nor the B-cell (CD19+) phenotypes. They were cytotoxic against the K562 cell line target. Although, qualitative assays have been widely applied, few quantitative assays have been developed to measure human NK function.

In Sato's experiment, fetal thymocytes of 16 to 22 gestational weeks were incubated with IL-15, IL-7 and SCF for two weeks. The cell colonies expressed CD56 but not CD3. They were also cytotoxic against K562 (Sato et al., 1999).

Another investigation demonstrated that the early human haemopoietic stem cell phenotype CD34+ CD3- CD8- CD56- yielded NK cell clones in the presence of rIL-15, suggesting an important role for this cytokine in development and maturation. The distinction between non-T derived NK and NKT-cells that have some of the hallmarks of thymus processed cells is unclear, and it has not been attempted to distinguish between these subsets in this study. The role of thymic NK is still unclear (Mrozek et al., 1996; Sivakumar et al., 1998; Yu et al., 1998; Punzel et al., 1999; Liu et al., 2000a).

NK cells do not express a functional T-cell receptor (TcR), except natural killer T-cells (NKT-cells) (see section-1.1.8), because they do not rearrange the α/β and γ/δ TcR genes line. However, NK cells may express limited transcripts of β and ζ proteins of the TcR complex associated with the Fc receptor (FcγRIII). FcγRIII (CD16) is the receptor for IgG_1/IgG_3 and stimulates the antibody-dependent cytotoxicity mediated pathway in NK cells. They also express FcRII (CD32) and FcμR that are involved in signal transduction and NK cell function. The TcR is not involved in NK cell recognition or cytotoxic activity, and recognition is not restricted through the major histocompatibility complex (MHC). However, recent studies show that NK cytotoxic function is abrogated when its receptors bind to MHC Class I molecules (Lanier et al., 1992; Bancroft, 1993; Warren et al., 1994). Since NK receptors recognise self-HLA Class I and are inhibited, they are called killer inhibitory receptors (KIR). NK cells are sensitive to

'missing-self ' MHC Class I molecules as they occur in virally infected or neoplastic cells. The lack of inhibitory signals via the KIR leads to killing of the tumour cell (Lanier, 2000b; Parham, 2000).

Typically human NK cells do not express CD3 but do express interleukin 2 receptor (IL-2R), CD16 and CD56, the latter being a major hallmark (Carson et al., 1994; Carayol et al., 1998). In humans, CD16, the low affinity receptor for IgG (FcγRIII), is also expressed on macrophages, mast cells and neutrophils. CD16 exists in two isoforms: a 56-60 KDa trans-membrane isoform (FcγRIIIA) expressed on the NK cell, monocytes and macrophages; and a 48 KDa isoform (FcγRIIIB) expressed exclusively on neutrophils. CD16 may also be expressed on CD3+ T-cells in certain individuals. CD56 is expressed on >95% of NK cells, NKT- cells and some mature human haematopoietic cells. CD56 is also expressed in adult neural, muscle and embryonic tissues. A number of tumour cell types are positive for CD56 including some myeloid leukaemia, myelomas, neuroblastomas, Wilm's tumour, and small cell carcinomas (Whiteside and Herberman, 1994; Schlossman et al., 1995; Barclay et al., 1997; Carayol et al., 1998; Godfrey et al., 2000). Activated NK cell express in addition, HLA-DR, CD69 (which acts as a signal-transmitting receptor involved in cellular activation), CD71 (transferrin receptor) and CD25 (IL-2Rα) (Whiteside and Herberman, 1994; Whiteside and Herberman, 1995b).

Furthermore, NK cells express cellular adhesion molecules (CAM) to bind to targets and extra-cellular matrix (ECM) affecting NK migration from the blood circulation to infection or tumour sites across the vascular endothelial cells. NK cells express β_1 integrins, which are cell surface receptor proteins for a variety of ligands especially VLA-4, VLA-5 (fibronectin receptor) and VLA-6 (laminin receptor). These facilitate NK cell binding to ECM and targets. β_2 integrins are involved in signal transduction and NK cell activation (Rabinowich et al., 1995; Whiteside and Herberman, 1995b).

NK cells are effector cells of the innate immune response and are important during the early stages of viral and intra-cellular bacterial infections

acting without the need for prior immunisation. They are activated by local signals upon interaction with other cells or extra cellular components. Functional assay systems, other than lysis of the K562 cell line target, have indicated that NK cells play a vital role in defence against intra-cellular bacterial infection, virus-infected and malignant cells (Bancroft, 1993; Biron et al., 1999). Patients with reduced or no NK cell activity have a high frequency of viral infections such as HSV, CMV, EBV, varicella and LCMV and malignant cells (Pross and Lotzova, 1993; Brittenden et al., 1996). NK cells have a minor role in defence against intra-cellular bacterial infections and antibody-dependent cell mediated cytotoxicity (ADCC). Other components of innate immunity such as monocytes/macrophages, PMN cells, complement and antibodies play a major role in defence against bacterial infections through opsonisation and/or phagocytosis (Klein and Horejsi, 1997; Janeway et al., 2001). Normal mice infected with lymphocytic choriomeningitis virus (LCMV) show peak NK cell activity on day three following infection, whereas, peak CD8+ T cell activity occurs on day seven (Su et al., 1994). Also, NK cells are involved in the defence against leukaemia (Silla et al., 1995).

NK cells may have a role in the regulation of haemopoiesis by producing a spectrum of cytokines such as colony-stimulating factor (CSF) and granulocyte/macrophage colony stimulation factor (GM-CSF). NK cells also activate PMN cells to kill Candida Albicans (Whiteside and Herberman, 1994; Warren et al., 1995; Biron et al., 1999). As they differentiate, human NK cells secrete inflammatory cytokines such as IFNγ, tumour necrosis factor alpha (TNFα), IL-3, IL-8, CSF and GM-CSF (Carson et al., 1995b; Kos, 1998; Biron et al., 1999). Also, they produce chemokines such as MIP-1α, MIP-β and lymphotactin (Biron et al., 1999; Lanier, 2000a).

Figure-1.1 demonstrates the NK cell development and cross talking between activated NK cells and macrophages in cytokine synthesis as part of innate immunity, as described in the literature.

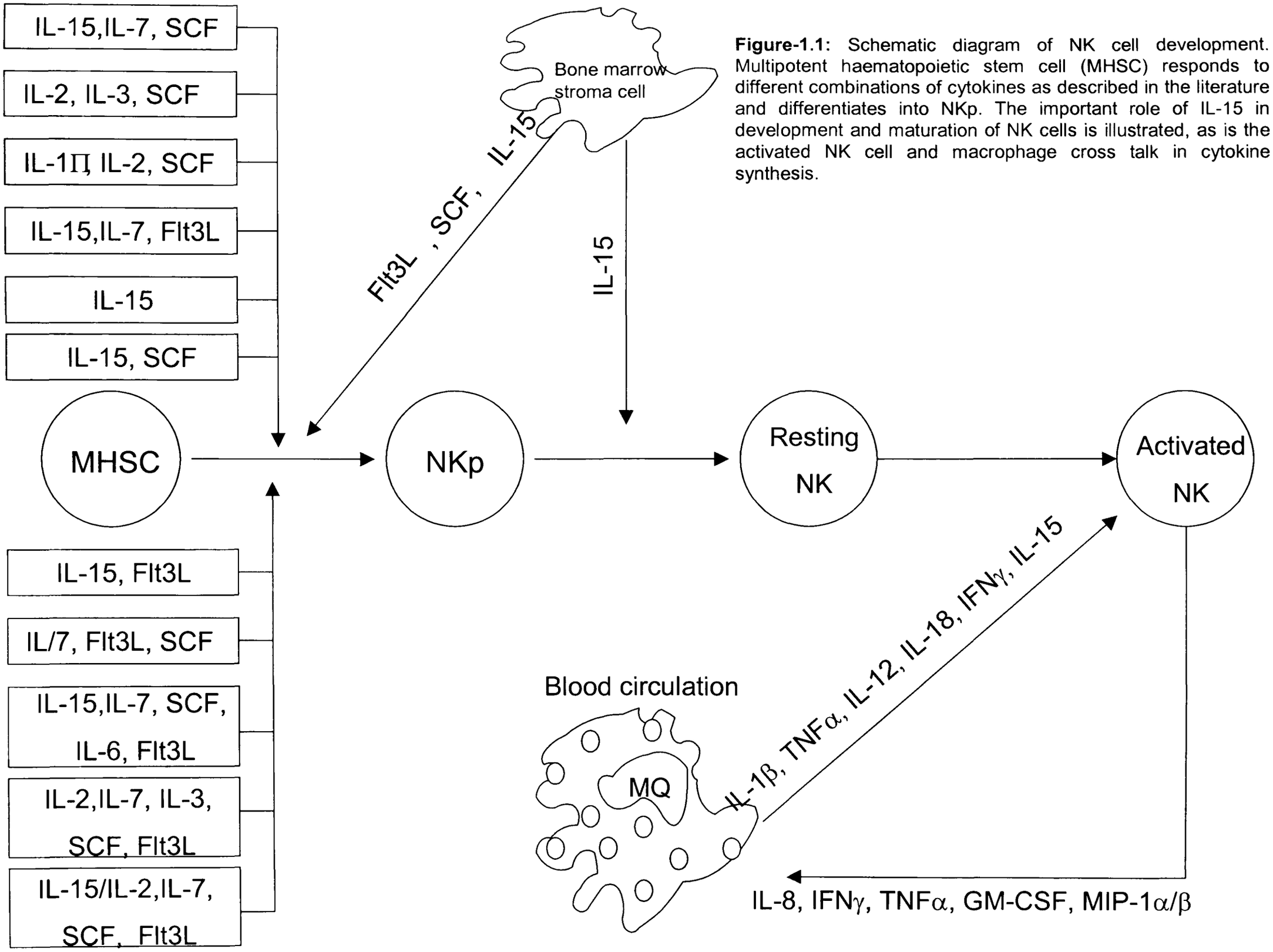

Figure-1.1: Schematic diagram of NK cell development. Multipotent haematopoietic stem cell (MHSC) responds to different combinations of cytokines as described in the literature and differentiates into NKp. The important role of IL-15 in development and maturation of NK cells is illustrated, as is the activated NK cell and macrophage cross talk in cytokine synthesis.

In certain culture conditions, NK cells may produce anti-inflammatory cytokines including IL-5, IL-10 and IL-13 (Warren et al., 1995; Mehrotra et al., 1998; Peritt et al., 1998; Hoshino et al., 1999b). In Warren's study (1995), NK cells produced IL-5 when stimulated with gamma-irradiated melanoma cells (MM-170) or human EBV-transformed B-lymphoblastoid cells (JY) and rIL-2. The IL-5 production was increased by IL-4, but inhibited by IL-12. In addition, IL-5 produced in freshly isolated NK cells in primary culture was higher than re-stimulated secondary culture. In Mehrotra's experiment (1998), freshly purified NK cells proliferated in response to IL-2 stimulation and produced IL-10. Adding IL-12 enhanced this phenomenon. But, IL-12 alone induced neither substantial proliferation nor detectable IL-10 production by fresh NK cells. Nonetheless IL-10 mRNA expression and protein syntheses were detected. By contrast in Peritt study (1998), NK cells cultured in the presence IL-12 produced IL-10, while these grown in IL-4 produced IL-5 and IL-13. In Hoshino's experiment (1999a), the production of IL-13 was observed in the presence IL-2. Finally, phorbol myristate acetate and ionomycin induce NK cells to produce IL-13 (Hoshino et al., 1999a; Hoshino et al., 1999b).

Figure-2.1 demonstrates the anti-inflammatory cytokines synthesis by NK cells in certain culture as described in the literature.

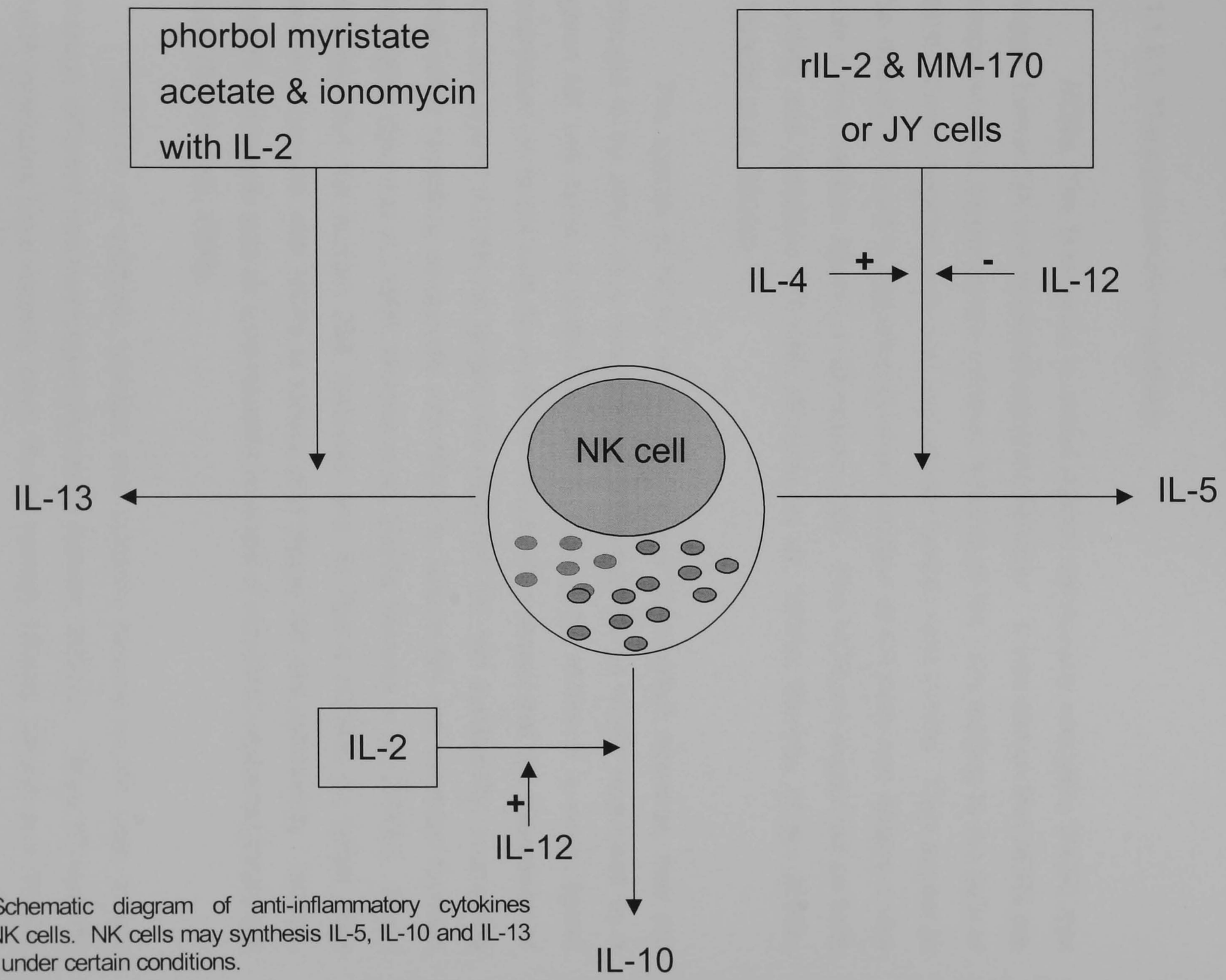

Figure-2.1: Schematic diagram of anti-inflammatory cytokines produced by NK cells. NK cells may synthesis IL-5, IL-10 and IL-13 when cultured under certain conditions.

1.1.2. Human NK cell receptors

A balance between two different receptors expressed on the NK cell's surface regulates the cytotoxic function of human NK cells.

1.1.2.1. The cytotoxicity receptors

NCRs. The first group is called natural cytotoxicity receptors (NCR) that trigger human NK cell mediated cytotoxic function. It was shown that NCRs are responsible for triggering the cytotoxic function of NK cells leading to the lysis of MHC Class I negative cells and xenogeneic tumour cells in-vitro. They appear to be directly involved in triggering cytotoxic function of NK cells and display a vital role in the reaction against most tumour cells. The NCR are expressed on both resting and activated NK cells (Moretta et al., 2000a; Moretta et al., 2000b; Moretta et al., 2000c).

The ligands of NCRs on target cells are unidentified, however, they are thought to be either as a complete set of ligands for all NCRs expressed by a given NK cell clone, a limited number of ligands for NCRs or a single ligand expressed on target cells for an NCR. It was also shown that the expression of the 2B4 ligand (CD48) on target cells triggered NK cell cytotoxicity, suggesting that other receptors co-operate with NCRs to induce NK cell cytotoxic function triggering (Sivori et al., 1999; Moretta et al., 2000a; Moretta et al., 2000b). Sivori showed that the human 2B4 molecule and its ligand (CD48) on target cells should cross-link with NCRs to induce and trigger NK cell cytotoxicity. Indeed, the 2B4 molecule acts as a co-receptor involved in non-MHC-restricted target cell lysis (Sivori et al., 1999).

Initiation of cytotoxic function and cytokine release in NK cells occurs through different non-rearranged receptors (Lanier, 2000b). Three NK-specific NCR molecules have recently been cloned namely NKp46, NKp30 and NKp44. NKp46 is involved in the recognition and lysis of HLA-Class I-unprotected allogeneic or xenogeneic cells. For example, murine target lysis by human NK

cells occurs through NKp46. Furthermore, NKp46 is the most important NCR since it plays a vital role in the recognition and lysis of tumour cells in man. NKp30 has the same surface pattern expression as NKp46, but some tumour cells are directly targeted through NKp30. NKp44 is expressed on IL-2 activated but not resting NK cells and it appears NKp44 can be measured as a specific marker for activated NK cells (Sivori et al., 1999; Moretta et al., 2000a; Moretta et al., 2000b).

It has also been reported that human NK cells recognise cell-surface glycosylphosphatidyl-inositol-linked molecules, called UL16-binding proteins (ULBPs). ULBPs ligate the NKG2D molecule that provides a positive signal for NK cytotoxic function, and cytokine and chemokine production including IFNγ, TNFα, GM-CSF, LTα/TNFβ and MIP-1β (Cosman et al., 2001). ULBPs are expressed on T-cell, B-cell, erythroleukaemia cell lines and some tissues including heart, brain, lung, liver, testis, lymph node, thymus, tonsil and bone marrow. Moreover, expression of ULBPs on cells that are naturally resistant to NK cell cytotoxic function (e.g. Daudi cells, a Burkitt's leukaemia/lymphoma cell line, that express MHC Class I molecules) become sensitive to NK killing (Cosman et al., 2001).

1.1.2.2. The inhibitory receptors

KIR. The first family of inhibitory receptors on NK cells are termed killer inhibitory receptors (KIR). This group of inhibitory receptors is functional in man but not rodent. Most people have different KIR genes not all of which appear inhibitory. The expression of KIR varies considerably among individuals although all KIR genes are present in the genome. It is assumed that when an NK precursor (NKp) is educated each NKp makes a random choice of which KIR genes it expresses. Ultimately, each NK cell expresses at least one KIR, having undergone allelic exclusion until it ends up with a self-HLA-specific KIR that is sufficient to prevent cytotoxic reactivity against normal self-cells (Reyburn et al., 1997; Ruggeri et al., 1999).

KIRs bind directly to MHC Class I molecules, and inhibit NK cell cytotoxic function signalling. However, the expression of MHC Class I molecules is frequently impaired in tumour and virus-infected cells that abrogate immunoreceptor tyrosine-based inhibition motifs (ITIM) inhibitory signalling and induce NK-mediated cytotoxicity. ITIM is an amino-acid stretch molecule with a tyrosine on its centre that present in the intra-cytoplasmic domain of inhibitory receptors (Blery et al., 2000).

In man KIRs are a family of immunoglobulin like (Ig-like) molecules (Ruggeri et al., 1999). The inhibitory receptors recognise different HLA Class I molecules such as HLA -A, B or C (Selvakumar et al., 1997). The KIR2D recognises certain HLA-C allo-types. KIR2DL1 recognises Cw1, 3, 7, 8 and KIR2DL2 recognises Cw2, 4, 5, 6 allo-types (Ruggeri et al., 1999).

KIR3DL1 recognises an epitope shared by HLA-Bw4 alleles and KIR3DL2 recognises HLA-A3 and HLA-A11. KIR3DL1 and KIR3DL2 contain three Ig domains (Ruggeri et al., 1999; Colonna et al., 2000).

CD94. The second family of inhibitory receptors includes CD94-NKG2, a lectin-like heterodimer identified in both man and rodent. The CD94-NKG2A isoform, is the only functional inhibitor and this recognises the monomorphic HLA-E molecule. The expression of CD94-NKG2A on NK cells in man is induced by IL-15 (Diefenbach and Raulet, 2001).

Ly-49. The third family of inhibitory receptors is Ly-49, a multigene family that encodes three different groups of inhibitory receptors and enables NK cells to distinguish target cells from self. The first inhibitory receptor group, discovered in rodent and called Ly49, binds directly to MHC Class I molecules (Karlhofer et al., 1992). Eight of ten different Ly49 homodimer receptors, identified so far in rodent, show inhibitory function (McQueen et al., 1999). In man only one non-functional molecular homology belonging to the Ly49 multi-gene family has been discovered (Westgaard et al., 1998).

Inhibitory receptors transduce signal(s) that inhibits activation signals. This is achieved via ITIM in their cytoplasmic tails. The phosphatases activated by ITIM inhibit signals derived from homologous activating motifs, called immunoreceptor tyrosine-based activation motifs (ITAM) with a high homology with ITIM (Blery et al., 2000). If ITIM are not activated, as in the case of a MHC Class I negative cell (e.g. K562), the ITAM signal the NK cell to kill the target. Thus, by the induction of inhibitory signals, normal cells are protected against NK-mediated lysis. By contrast, NCR induces a signal that triggers cytolytic activity mediated by perforin and granzyme release that leads to lysis or apoptosis of the target cell (Pende et al., 1999; Moretta et al., 2000b). The ontogeny of NK cell is not fully understood. This process involve the differential expression of both cytotoxicity and inhibitory receptors on individual NK cells (Raulet et al., 2001).

Figure 3.1 demonstrate interaction between MHC Class I molecules and some receptors (Trowsdale, 2001). Figures 4.1 and 5.1 demonstrate known ITIM-bearing molecule and interaction with KIRs (fig-4.1) and oligomeric activating receptor complexes expressed on NK cells (fig-5.1) (Blery et al., 2000).

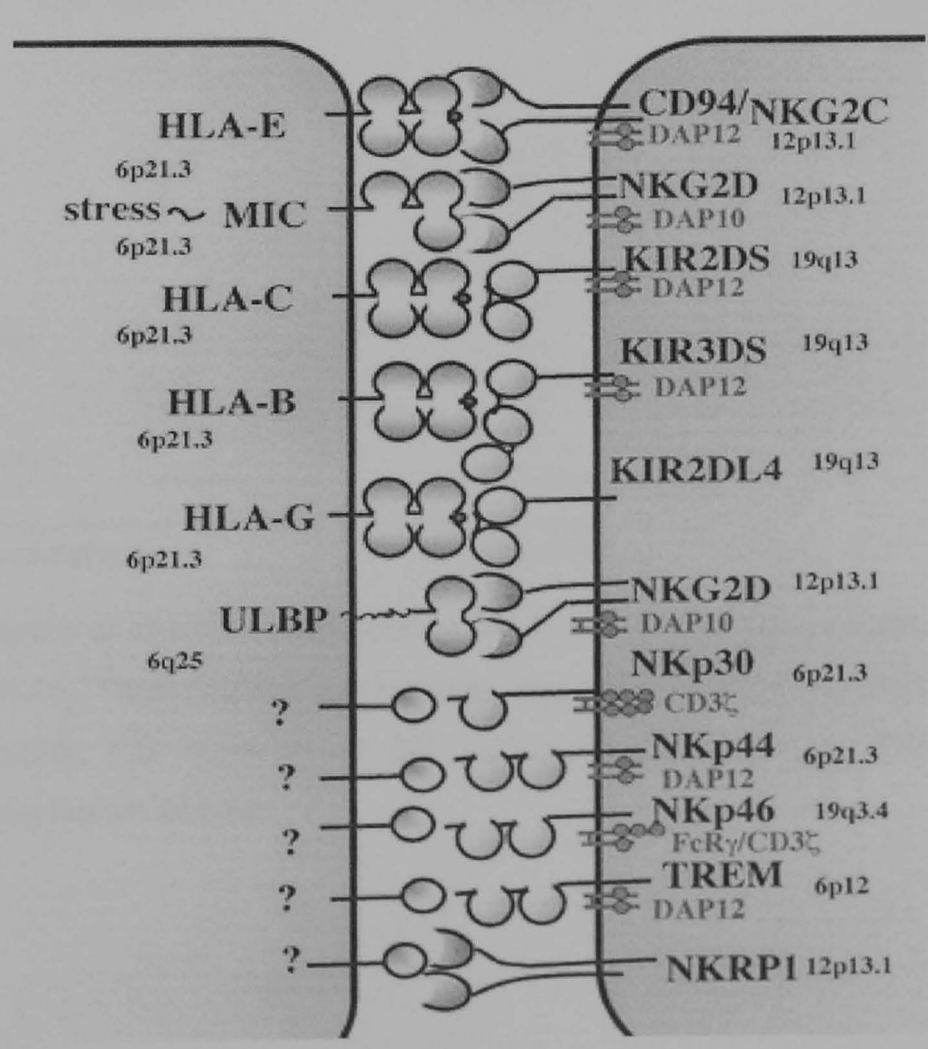

Figure 3.1: Schematic diagrams of some of the cytotoxicity receptors are shown along with their chromosomal position and Class I ligand. NKG2D is functional in rodent and man, and KIR2DL4 has not been confirmed in man.

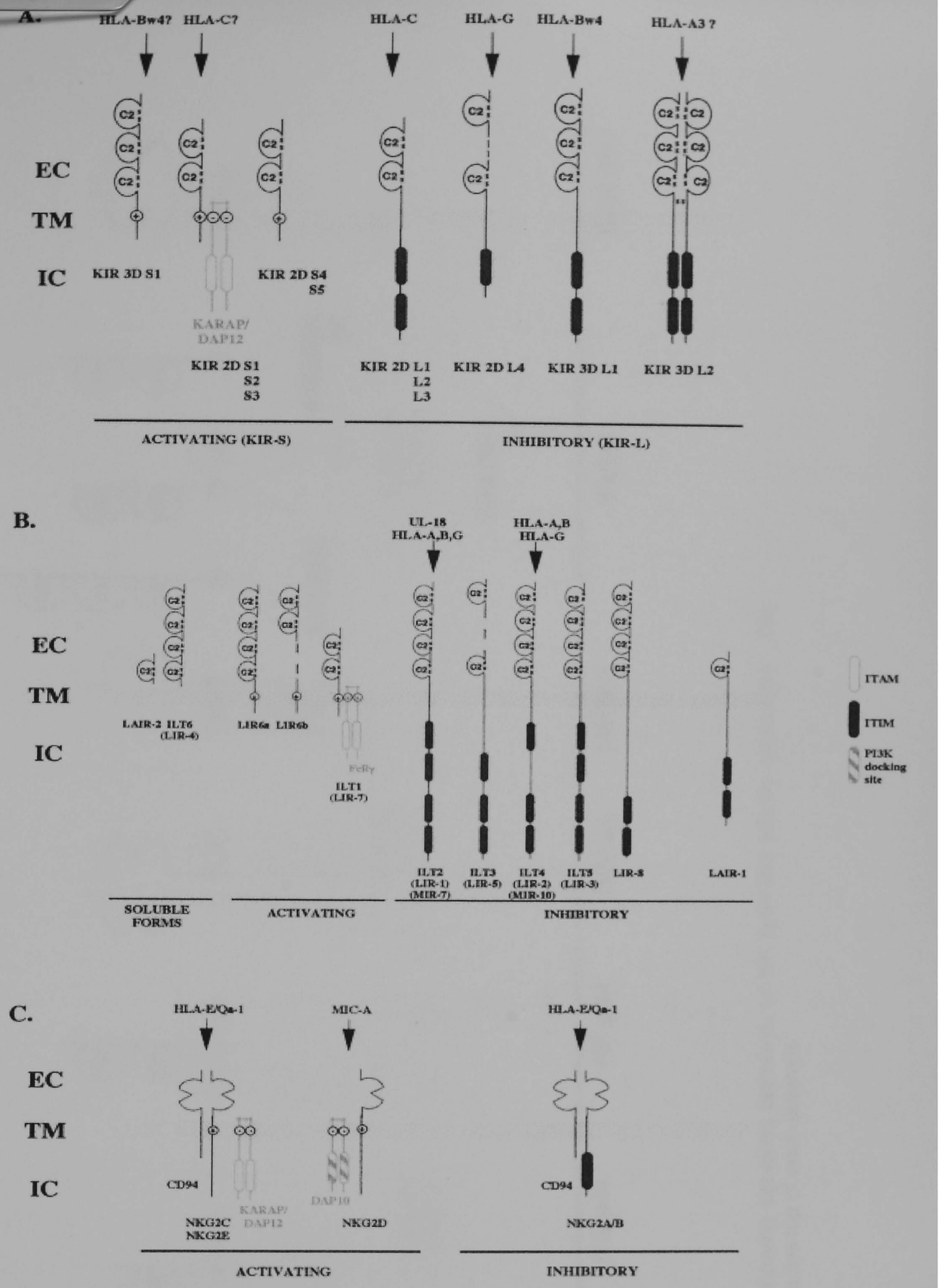

Figure 4.1: Schematic diagram of interaction between ITAM and ITIM-bearing receptors and their ligands (HLA-Bw4 etc) in man. Three families are included: **(A)** killer inhibitory receptors (KIRs)*, (B) monocyte Ig-like receptor, (C) CD94-NKG2. **EC:** extracytoplasmic domain, **TM:** transmembrane domain, **IC:** intracytoplasmic domain.

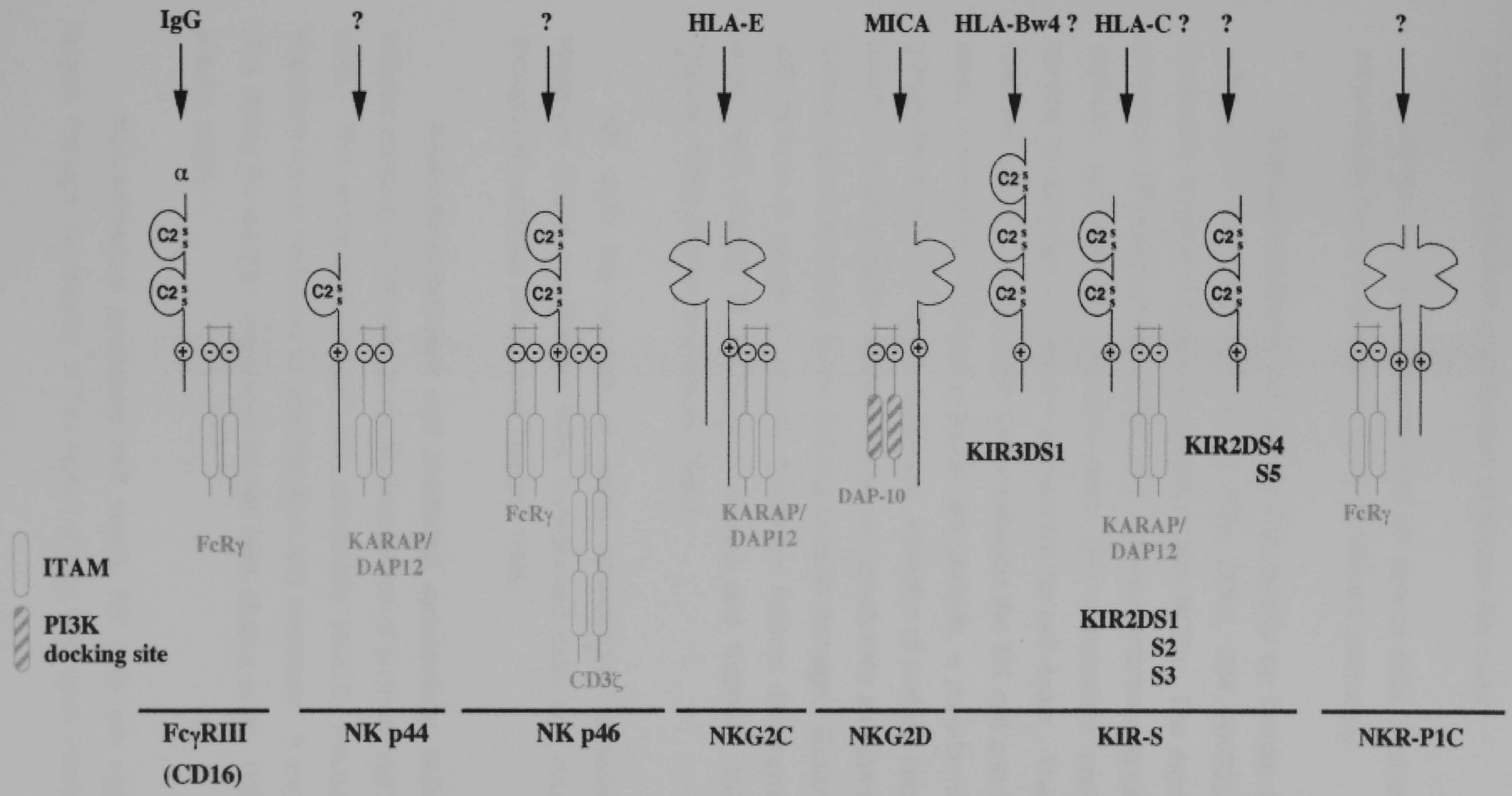

Figure 5.1: Schematic diagram showing signalling pathways on NK cells that initiate cytotoxicity. The ligand of each receptor is shown at the top of each diagram.

1.1.3. The cytotoxicity mechanism of human NK cells

Human NK cells recognise and kill tumour cells, viral cells or cells with intra-cellular bacteria through a variety of different pathways.

Granule-mediated cell death. Cytotoxicity by human NK cells follows adhesion to the target cell via the CD2, CD69, 2B4, NKG2D-DAP10, LFA-1 (leukocyte function antigen-associated-1) and NCRs. The expression of CD69 precedes CD25 expression on NK cells. Trans-membrane signalling through this pathway activates phosphatidyl-inositol (PI) metabolism and calcium influx leading to secretion of granules responsible for cell death. These granules are released into the intra-cellular space between the NK cell and its target. The major components of these granules are perforin, a pore-forming protein, and granzymes a family of serine proteases. Insertion of perforin into the lipid bilayer results in polymerisation of the membrane constituents and the formation of the tubular trans-membrane pores causing osmotic damage. Granzymes synergise with perforin to trigger a caspase dependent internal disintegration pathway in target cells leading to apoptosis (Tschopp and Nabholz, 1990; Smyth and Trapani, 1995; Warren and Smyth, 1999).

NK cells are capable of lysing intra-cellular bacteria-infected cells, malignant and/or transformed cells, transplanted cells and virus-infected cells through this pathway (Smyth and Trapani, 1995).

Antibody-dependent cell mediated cytotoxicity (ADCC). Another effector pathway of NK cells is via the recognition of antibody (IgG)-coated target cells. This antibody-dependent cell cytotoxicity (ADCC) mechanism plays an important role in response to viral infection and neoplasia. It involves the CD16 (Fcγ RIIIA) Fc receptor expressed on NK cells (Sulica et al., 1996; Warren and Smyth, 1999).

Non-secretory apoptotic cell death. NK cells are capable of killing targets through expressing of Fas ligand (FasL) and upon interaction with Fas

(CD95/APO-1) on target cells, signal the pathway leading to apoptosis. The expression of FasL on activated NK cell is remarkably enhanced (Puppo et al., 2000; Puppo et al., 2001).

Secreted TNFα by NK cells engages the tumour necrosis factor receptor (TNFR) on target cells and induce apoptosis cell death (Vujanovic et al., 1996).

Pro-inflammatory reaction. Activated NK cells produce IFNγ, TNFα, GM-CSF, IL-3 and IL-8 thereby inducing an inflammatory cascade. IFNγ released rapidly before adaptive immunity has developed is essential to first line defence and plays a pivotal role in suppressing or slowing infection (Scharton and Scott, 1993; Orange and Biron, 1996; Biron et al., 1999). Secreted IFNγ, IL-2 and IL-15 are the main regulatory signals for NK cell cytotoxic function (Cousens et al., 1997; Lin et al., 1998)

Other cytokines involved are TNFα and IL-1 act synergistically with IL-2 to activate large granulocyte lymphocytes. IL-12 stimulates IFNγ production and induces cytotoxicity activation. IFNα augments NK cell function thereby affecting the kinetics of cytotoxicity (Cousens et al., 1997; Lin et al., 1998).

1.1.4. Interleukin-2 and NK cells

Cytokines are hormone-like glycoproteins produced by a wide variety of cells involved in intercellular communication. They affect the activation, differentiation and proliferation of cells involved in haemopoiesis, immunity, and the inflammatory processes. During differentiation and proliferation of haemopoietic stem cells, cytokines affect progenitor cell-cycling and lineage specific differentiation (Mosmann et al., 1997; Annunziato et al., 1999).

In-vivo, IL-2 is thought to be only transiently produced by antigen-activated T-cells, which in turn express high affinity IL-2 receptor (IL-2R). Activated T-cells release IL-2, a 15-kD glycoprotein belonging to the helical-cytokine family. IL-2 is able to drive NK and T cells to activation, proliferation and cytokine induction. IL-2 causes NK cells to become lymphokine-activated killer (LAK) cells that are active against certain NK resistant tumour cells. IL-2 stimulates NK cells through the α, β and γ sub-units of the IL-2R expressed on the surface of NK cells. IL-2 binds to α sub-unit with low affinity binding, to the β and γ sub-units with intermediate affinity binding and to the triplet α, β and γ with high affinity. The IL-2Rβ (CD122) chain is shared between the IL-2 and IL-15 receptors only, whereas the IL-2Rγ (CD132) common chain (γ_c) is shared by IL-2, IL-4, IL-7, IL-9 and IL-15 receptors (Voss et al., 1992; Shi et al., 1997; Waldmann et al., 1998).

IL-2 can drive CD34+/DR- haemopoietic progenitor cells to differentiate into NK cells with a characteristic CD3- CD56+ phenotype (Miller et al., 1998; Miller et al., 1999).

IL-2 also stimulates a number of other cells, including B-cells in the production of opsonising antibodies, proliferation and activation, and monocytes. It has been shown that IL-2 is a powerful activator of monocytes, leading them to express tumouricidal and microbicidal activity and increasing the synthesis and release of hydrogen peroxide and superoxide (Badolato et al., 1997; Bosco et al., 1997).

1.1.5. Interlukin-15 and NK cells

IL-15 is a 14-15-kD glycoprotein that, like IL-2, belongs to a four alpha helix bundle family of cytokines. It was recently discovered and cloned in 1994 in the culture supernatants from simian kidney epithelial cell lines that activated human monocyte/macrophages (Carson et al., 1994; Carson et al., 1995b). Bone marrow stromal cells, dendritic cells and keratinocytes, all produce IL-15. IL-15 mRNA has been detected in the placenta, skeletal muscle, kidney and synovial-derived cells in rheumatoid arthritis patients (Blauvelt et al., 1996; Doherty et al., 1996; McInnes et al., 1996; Jonuleit et al., 1997).

Recent reports suggest that IL-15 shares many of the biological properties of IL-2, despite the absence of sequence homology. The interlukin-15 receptor (IL-15R) has a unique α chain, but shares the β and γ chains of the IL-2R. Since signals are only transduced via the β and γ chains this accounts for the shared activity observed between these two cytokines, whereas the IL-15Rα chain is only responsible for specific and high-affinity binding of IL-15. Signals received by IL-15R cause NK cells to produce IFNγ, GM-CSF and TNFα (Gosselin et al., 1999; Waldmann and Tagaya, 1999). Mice lacking the IL-2 gene develop NK cells, whereas mice and humans lacking the γ sub-unit of the IL-2R have no NK cells. In addition, mutations in human IL-2Rγ_c appears responsible for the X chromosome-linked immunodeficiency disease (SCIDX) characterised by a defect in T-cell differentiation and the lack of NK cells (Noguchi et al., 1993; DiSanto et al., 1995). It was also reported that IL-15Rα-knockout or IL-2/IL-15Rβ-knockout mice have no NK, NKT- and $\gamma\delta$T-cells, which are important in innate immunity (Liu et al., 2000a). Furthermore, in an in-vivo study it has been shown that mice treated with the isotope strontium 89 (^{89}Sr) have no NK cells because of impairing of IL-15 production within the bone marrow microenvironment (Colucci and Di_Santo, 2000). IL-15 is required for induction of CD56 and CD7 in early human NK cell development (Mrozek et al., 1996; Carayol et al., 1998).

In-vitro, IL-15 as well as IL-2, enhances the cytotoxicity and maturation of neonatal NK cells activity against K562 and ADCC function, as well as the percentage of CD56+CD16+ cells (Gaddy and Broxmeyer, 1997; Nguyen et al., 1998). IL-15 was also shown to stimulate the expansion of HIV-specific cytotoxic T-cells and enhance NK cell cytotoxic function through ADCC pathway in AIDS patients (Lin et al., 1998; Nguyen et al., 1998).

Table-1.1 shows the cell markers expressed on multi-potent haemopoietic stem cell (MHSC), NK precursor (NKp), resting NK and activated NK cells as described in the literature.

Table-1.1: Cell markers expressed during the maturation of NK cell

Cell markers	MHSC	NK precursor	Resting NK cell	Activated NK cell
CD2	-	+	+	+
CD3	-	-	-	-
CD4	-	-	-	-
CD5	-	-	-	-
CD7	-	+	+	+
CD8	-	-	-	-
CD14	-	-	-	-
CD15	-	-	-	-
CD16	-	-/+	-/+	-/+
CD25 (IL-2Rα)	-	-	-	+
CD32	-	+	+	+

CD33	-	+	+	-
CD34	+	-/bright	-	-
CD38	-	+	+	+
CD44	-	+	+	+
CD45	+	+	+	+
CD56	-	+	+	+
CD57	-	+	+	+
CD69	-	-	-	+
CD94-NKG2	-	+	+	+
CD122(IL-2/1L-15Rβ)	(+)?	+	+	+
CD132(IL-2/IL-15Rγ)	(+)?	+	+	+
IL-15Rα	(-)?	+	+	+
HLA-DR	-	-	-	+
Flt3	+	+	(+)?	(+)?
c-kit (CD 117)	+	+	(+)?	(+)?
KIRs	-	(+)?	+	+
NKp30	-	-	-	+
NKp44	-	(+)?	+	+
NKp46	-	(+)?	+	+
2B4	-	+	+	+
VLA-4	-	(+)?	+	+
VLA-5	-	(+)?	+	+

VLA-6	-	(+)?	+	+
FasL	-	+	+	+
Ly49	-	(+)?	+	+

1.1.6. Prostaglandin E_2 (PGE_2) and NK cells

Prostaglandin E_2 (PGE_2) is a major contributor to the production and maintenance of immunosuppression after major, haemorrhage and injury, leading to increased infectious morbidity and mortality in trauma patients. Deleterious effects of PGE_2 on NK cytotoxic function and cytokine production, have been shown (Menetrier_Caux et al., 1999; Deichman et al., 2001).

Rees and Platts showed that NK cytotoxic function in both whole blood and isolated PBMC was significantly decreased in the presence of PGE_2 (Rees and Platts, 1983). Furthermore, a significant decline in cytotoxic function and IFNγ production occurred in IL-15-activated (200 ng/ml IL-15 for 2 days) human NK cells in the presence of PGE_2 (10-200 ng/ml). This suggested that PGE_2 down-regulated surface expression of γ_c-chain on NK cells leading to suppression of IL-15 activation (Joshi et al., 2001). It was reported that the proliferation of lymphokine-activated killer (LAK) cells induced by IL-2 was inhibited by PGE_2 (Wang et al., 1997)

In animals hepatic NK function was inhibited by PGE_2, at 10 -20 ng/ml. By contrast, there was a significant increase in NK function prostaglandin F2 alpha (PGF_2 -α) by at 50-100 ng/ml (Liu et al., 2000b). PGE_2 inhibitors, such as indomethacin and ibuprofen abrogate the suppressive effects of PGE_2 on NK cytotoxic and ADCC functions (Lo et al., 1998; Rhind et al., 1999).

The mechanism by which PGE_2 inhibits is not fully understood, however, binding between activated NK cells and target cells is inhibited by PGE_2. Another possibility is that cAMP concentration increases leading to suppression of cytolytic protein release and NK cell-mediated cytotoxicity (Kim et al., 1999).

1.1.7. The Lymphokine-Activated Killer (LAK) cell

NK cells are able to recognise and killing tumour cells, thereby playing an important role in immune defence against malignancy. Incubation of normal peripheral blood lymphocytes with IL-2 (1000 U/ml) from patients with neoplasia leads to the generation of lymphokine-activated killer (LAK) cells that in the short-term can lyse fresh tumour cell targets. Indeed, the in-vitro culture of human peripheral blood lymphocytes in high concentrations of IL-2 results in the generation of cytotoxic cells that lyse tumour cell targets that are normally resistant to NK cell lysis through a mechanism independent of MHC restriction. LAK cells have the phenotype CD2+, CD3-, CD11+, CD14- and CD56+. These LAK cells are capable of binding to capillary endothelial cells and migrating into solid tumour tissue. However, it was reported that treatment with low concentration of IL-2 ($2x10^5$ U/m^2 per day) did not lead to an appreciable clinical response against the tumour (Whiteside and Herberman, 1994; Barton et al., 1995; Brittenden et al., 1996).

Nonetheless, several observations have indicted that in-vitro and in-vivo activated NK cells are able to move in to solid tissues, localise to the site of metastases and kill tumour cells. Treatment using IL-2 alone, or IL-2 in conjunction with LAK cells, has also been shown to mediate disease regression in selected patients with advanced cancer and even leukaemic relapse after autologous bone marrow transplantation. In-vitro LAK cell activity could be enhanced by adding IL-12, IL-7, and IL-15 (Sondel et al., 1992; Vujanovic et al., 1995; Okada et al., 1996; Tomita et al., 1998).

1.1.8. Human Natural Killer T-cells (NKT-cells)

In 1978 NKT-cells were characterised by the immunophenotype, CD4-CD8- or CD4+CD8- low TcR and NK1.1+ cells (Fowlkes et al., 1978; Budd et al., 1987). The term, NKT-cells, was recently given to them (Godfrey et al., 2000). The NKT-cell subset expresses some common characteristics of both T- and NK cells by having CD3 and TcR α/β, as well as CD56, CD1d and KIR. The important characteristics of NKT-cells are expression of TcR, CD1d and production of IFNγ and IL-4 (Leite_de_Moraes and Dy, 1997; Godfrey et al., 2000).

They share functional activity with T-cells including secretion of both Th_1 and Th_2 cytokines, with particularly high levels of IL-4 and IFNγ after stimulation through MHC Class I molecules, and the NK1.1 molecule. They are also able to lyse target cells that have lost, or express insufficient MHC Class I molecules. KIR and NK1.1 expression suggests that NKT-cell populations identify and lyse targets in the same way as NK cell (Storkus et al., 1992; Arase et al., 1994; Leite_De_Moraes et al., 1998)

Some NKT-cells develop along extrathymic pathways and are able to participate in the innate immune response to tumours whereas other NKT-cells are thymus-dependent (Yoshimoto and Paul, 1994; Hammond et al., 1999). NKT-cells may play a key role in the regulation of anti-microbial immunity especially against mycobacterium, HIV and salmonella (Jason et al., 2000).

1.2. Adaptive immunity

Adaptive immunity is an acquired or "educated" response to pathogens or other foreign particles during the lifetime of an individual. Its origins coincide with the appearance of vertebrates and the acquisition of RAG. RAG allows for somatic mutation amongst the genes responsible for immunoglobulin (Ig) and TcR specificity. Ig and TcR genes are assembled by rearrangement of antigen receptor gene segments during lymphocyte development. Thus vertebrates not only have the benefit of innate immunity but also an immune system that can adapt to the continuous challenge posed by evolving pathogenic micro-organisms (Akamatsu and Oettinger, 1998; Landree et al., 1999).

In many cases an adaptive immune response to a pathogen confers life-long protection. Adaptive immunity has immunologic memory, which distinguishes it from innate immunity. It is initiated by antigen-presenting cells (APC), lymphocytes and various cytokines. Two major lymphocyte populations participate in adaptive immunity, T-cells and B-cells. T-cells express CD3, TcR and when activated, IL-2R. T-cells recognise peptides that have been processed and presented by APC in the context of self-MHC. T-cells are derived from multipotent haemopoietic stem cells in bone marrow but develop exclusively along thymic pathways. T-cell subtypes are CD4+ that recognise MHC Class II molecules or CD8+ that recognise MHC Class I molecules. Either may express $\alpha\beta$ or $\gamma\delta$ TcR. CD4+ T-cells are mostly helper cells that activate other cells such as CD8+ T-cells and B-cells, whereas CD8+ T-cells are mostly cytotoxic cells that kill intra-cellularly infected or neoplastic cells. In contrast to T-cells, B-cells originate and develop in bone marrow. They recognise the antigens via their B-cell receptor complex, which consist of an immunoglobulin (Ig) member. Each receptor is able to recognise and bind to a unique antigen. After binding, the B-cell ultimately differentiates into plasma cells that secrete specific antibodies (Klein and Horejsi, 1997; Roitt and Rabson, 2000; Janeway et al., 2001).

1.2.1. Pro and anti-inflammatory reactions

In 1986, Mosmann and his colleagues hypothesised that murine CD4+ helper T-cell clones could be characterised in-vitro according to the patterns of cytokines they synthesised thereby giving rise to a unifying concept called *"The Th_1/Th_2 Paradigm"* (Mosmann et al., 1986).

Thus CD4+ T-cells were segregated into two functional subsets: T-helper 1 (Th_1) and T-helper 2 (Th_2). Th_1 cells secrete pro-inflammatory cytokines such as IFNγ and IL-2. Th_2 cells secrete anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 (Cher and Mosmann, 1987; Coffman et al., 1987). In 1991 human Th_1 and Th_2 were isolated by Romagnani (Romagnani, 1991a).

Pro-inflammatory cytokines induce macrophage activation, cell cytotoxicity, and cell mediated immunity. They play a vital role in the eradication of infectious agents, including intracellular pathogens. Anti-inflammatory cytokines suppress phagocyte-dependent inflammation and promote strong antibody responses through B-cell activation. The anti-inflammatory cytokines include IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. They are associated with humoral immunity and allergic responses. They act on B-cells causing them to activate, proliferate and differentiate into antibody producing plasma cells. Type 0 (Th_0) cells are a third subset of T-cells that may differentiate into both Th_1 or Th_2 types, and may represent a distinct subset of differentiated precursor T-cells. The differentiation pathway selected by this subset depends on the cytokines in the microenvironment. Another subset is called Th_3. Cells of this subset produce high amounts of transforming growth factor (TGF)-β (Mosmann et al., 1997; Lucey, 1999; Romagnani, 2000).

Several factors influence differentiation along the Th_1 and Th_2 pathways, including the cytokine microenvironment, antigen dose, secondary signalling pathways, individual genetic background and antigen-presenting cells (Oriss et al., 1997; Del_Prete, 1998; Annunziato et al., 1999). Evidence suggests that IFNα and IFNγ increase IL-12 production by macrophages and maintain

expression of functional IL-12 receptors on Th cells and as such are the most powerful cytokines promoting differentiation of the uncommitted Th_0 cells along the Th_1 pathway. The most important cytokine to trigger differentiation of Th_2 is IL-4 (Mosmann et al., 1997; Morel and Oriss, 1998; Lucey, 1999).

Cytokines released by Th_1 and Th_2 cell subsets are involved in different strategies of protection against pathogens, and may also play a role in some pathological conditions. Studies in humans and gene-targeted mice have clearly shown that Th_1-dominated responses are effective in protection against several microbes, and usually result in their clearance. However, if the pathogen persists, the ongoing Th_1 responses may result in inflammatory tissue damage. Cytokines other than those produced by Th_1 cells, play a role in defence against intracellular pathogens for which Th_1 responses are not inherently protective like the IFNγ production during viral infections (De_Vita et al., 1998; Del_Prete, 1998; Romagnani, 2000).

1.2.2. Interferon gamma (IFNγ)

The interferon family is divided into two types based on the source of secretion. Type one interferon's, including IFNα and IFNβ are secreted by virus-infected cells and play a vital role as antiviral agents. Envelopes of virus, double-stranded RNA, bacteria and tumour cells induce their production (Farrar and Schreiber, 1993).

Type two includes IFNγ, which is a 35-kDa protein and was identified for the first time in 1965 as an antiviral agent in PHA-activated lymphocyte supernatants. Human IFNγ is secreted by activated Th_1, NK, NKT- and dendritic cells. IFNγ is a strong anti-viral and anti-tumour agent, also acting as an immunomodulator during the immune response by switching naive CD4+ T-cells to differentiate along the Th_1 pathway (Schreiber and Farrar, 1993; Coughlin et al., 1998).

Human IFNγ gene and IFNγ receptor (IFNγR) are located on chromosome 12 and 6 plus 21, respectively. Its receptor has two chains the first of which is a 90-kDa, α-chain receptor (IFNγR1) encoded on chromosome 6 and responsible for ligand binding, ligand trafficking through the cell and signal transduction. The second chain is a 62kD, β-chain encoded by on chromosome 21 and also required for signal transduction after ligand binding. Signal transduction via both chains is required for activity (Pestka, 1997).

IFNγ is essential for the activation, proliferation, differentiation and cytotoxicity of T-, NKT- and NK cells. It stimulates innate cell-mediated immunity via NK cells and NKT-cells and adaptive immunity based on the recognition of cell surface-bound antigen in association with MHC molecules. IFNγ has a negative effect on the proliferation and differentiation of Th_2 cells and inhibits the growth of Th_2 clones in-vitro. IFNγ production by NK cells is extremely fast and provides a reliable source of pro-inflammatory cytokine upon encountering an infectious agent or neoplastic cell before clonal expansion of adaptive immunity. IFNγ indirectly suppresses B-cell proliferation via IL-4 inhibition. By contrast, it

induces the expression of IL-2R on the surface of T-cells. IFNγ up-regulates expression of MHC molecules especially Class II thereby amplifying the antigen presentation pathway (Warren et al., 1996; Fernandez et al., 1999; Tanaka et al., 2000).

IFNγ is the most important macrophage-activating factor. Activated macrophages kill some of the intra- and extra-cellular parasites, as well as malignant cells. IFNγ synergises with TNFα and β to inhibit the proliferation of various cell types. Also, it induces oxygen and nitrogen anti-microbial effects on macrophages by inducing those enzymes that are involved in substrate and cofactor synthesis of iNOS (inducible nitric oxide synthase). iNOS promotes the production of reactive oxygen intermediates especially nitric oxide (NO) involved in killing of targets (Daubener and MacKenzie, 1999; MacKenzie et al., 1999).

IFNγ secretion by NK cells can be induced by IL-12, IL-15, IL-2, IL-18 (originally called IGIF or IFNγ inducing factor), epidermal growth factor (EGF), IL-1α and IL-1β. The most important cytokine, which stimulates IFNγ production by NK cells is IL-12 in collaboration with IL-18. Activated macrophages, keratinocytes and Kupffer cells secrete IL-18. It is capable of inducing IFNγ production by NK cells without collaboration with IL-12, whereas with IL-12, IL-18 induces IFNγ production by T-cells (Yoshimoto et al., 1998; Braun et al., 2000; He et al., 2000). IFNγ production can be inhibited by TGF-β, vitamin D3, cyclosporin A (CsA) and IL-10 (Biron et al., 1999; He et al., 2000). It has also been shown that IFNγ inhibits the production of IL-4 by Th_2 clones (Hoshino et al., 1999b).

A cultured NK cell line produces IL-13 and IL-5, suggesting that NK cells may produce IL-13 and IL-5 in the absence of IFNγ (Warren et al., 1995; Hoshino et al., 1999b). Hoshino and his colleagues suggested that the absence of IFNγ may favour the differentiation of naïve Th_0 cells to Th_2 cells that produce anti-inflammatory cytokines (Hoshino et al., 1999a; Hoshino et al., 1999b)

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1.2.3. Interleukin 10 (IL-10)

IL-10 is an 18.5-KDa protein containing 160 amino acids, which was first described as cytokine synthesis inhibitory factor (CSIF). As with IFNγ, IL-10 is an acid-sensitive, non-covalent homodimer of two interpenetrating polypeptide chains. There are two types of IL-10 receptor. The first is characterised as the α-chain (hIL-10Rα) expressed on most haemopoietic cells, T-cells, monocytes/macrophages. The second is the β-chain (hIL-10Rβ) expressed on the most cells and tissues. However, the structure and the signalling of IL-10R are not fully understood. Human IL-10 is secreted by CD4+ and CD8+ T-cells, monocytes/macrophages, B-cells, eosinophils and epithelial cells (Kotenko et al., 1997; Ding et al., 2000b). In mice, IL-10 is produced and released by Th_0 and Th_2 cells (de_Waal_Malefyt et al., 1991a; de_Waal_Malefyt et al., 1995).

Human IL-10 acts as an anti-inflammatory cytokine inhibiting the secretion of pro-inflammatory cytokines such as IL-1α/β, IL-8, IL-12, IL-18, TNFα, and G-CSF or GM-CSF by activated monocytes/macrophages. IL-10 is activity antagonised by IL-4. It also prevents the release of free oxygen radicals and nitric oxide-dependent microbicidal actively of macrophages and neutrophils (de_Waal_Malefyt et al., 1991b; Groux et al., 1998).

Soluble IL-10 reduces antigen-presenting cell function of monocytes/macrophages by down-regulating the expression of Class II MHC. It also down-regulates the expression of MHC Classes I and II on tumour cells, effectively interfering with HLA associated activation of T-cells. It inhibits T-cell proliferation by negatively regulating pro-inflammatory cytokine production by Th_1 cells (de_Waal_Malefyt et al., 1991a). In addition, it down-regulates the expression of CD54, CD80, CD86, and the LPS signal-transducing receptors, which are important molecules for T-cell activation. In contrast IL-10 increases CD16 and CD64 (Fcγ RI) expression on monocytes (Groux et al., 1998). IL-10 is a specific chemo-attractant for human T-cells (Matsuda et al., 1994). IL-10 via down-regulates the cyclooxgenase-2 (COX-2) pathway thereby inhibiting PGE_2 production (Moore et al., 2001).

IL-10 activates NK cells through increased sensitivity to IL-2 leading to increased cytotoxicity and cytokine release (Mosmann and Sad, 1996; Warren et al., 1996). In 1995, Carson et al showed that overnight incubation of NK cells with IL-10 significantly increased NK cell cytotoxic function in the presence of IL-2. They also showed that IL-10 with IL-2 enhanced the secretion of IFNγ, TNFα and GM-CSF by NK cells. There was no effect of added IL-12. Finally, they confirmed that human NK cells express IL-10R message as mRNA (Liu et al., 1994; Carson et al., 1995a).

IL-10 may also indirectly suppress IFNγ production by NK cells by inhibiting its induction by IL-12 (Romagnani, 2000). In an animal experiment by Fujii et al, IL-10 administration given after a booster vaccine enhanced anti-tumour activity and vaccine efficacy. In a clinical study by Lauw et al it was shown that rhIL-10 administration given after LPS significantly increase IFNγ release and NK activation (Lauw et al., 2000; Fujii_Si et al., 2001).

1.2.4. Interleukin 4 (IL-4)

Human IL-4 was recognised in the early 1980s as a B-cell growth factor (BCGF) (Leanderson et al., 1982; Muraguchi et al., 1982). After cloning the cDNA of this cytokine in mice and humans in 1986, it was termed IL-4. The human IL-4 genes are located on chromosome 5 in man and chromosome 11 in mouse. The molecular weight of human IL-4 is between 15 and 19 KDa, and it contains 129 amino acids (Mingari et al., 1986; Yokota et al., 2000).

IL-4 induced signal transmission is initiated by two receptors; IL-4R1 composed of IL-4Rα-chain plus common γ-chain, and IL-4R2 composed of IL-4Rα-chain plus IL-13Rα-chain. However, IL-4 has only a low degree of sequence homology with IL-13 (Nelms et al., 1999). Activated Th_2 cells secrete human IL-4. Basophils and mast cells also produce IL-4. IL-4 is a multifunctional cytokine that regulates proliferation and differentiation of B-cells, T-cells and monocytes. IL-4 was shown to have negative effects on NK cells by inhibiting genes involved in NK cell function. This effect is in contrast to the positive effects of IL-2 and IL-15 on NK cells. IL-4 inhibits IL-10 production by monocytes (Romagnani, 1991b; Nakamura et al., 1997). It also down-regulates other monocyte cytokine production and function. IL-4 is involved in the development of humoral immunity by inducing B-cell proliferation and induction the production of IgM, IgG_4 and IgE (Del_Prete, 1998; Romagnani, 2000).

Human IL-4 is the most potent cytokine directing the development of Th_2 cells, resulting in the release of the anti-inflammatory cytokines IL-5, IL-6, IL-10 and IL-13. These act as growth/differentiation factors for B-cells, eosinophils and mast cells and inhibit some macrophage functions. In the absence of IL-4 production during the early immune response, naïve T-cells are obliged to differentiate along the Th_1 pathway by IL-12 and IFNγ (Oriss et al., 1997; Romagnani, 2000).

1.3. Major Histocompatibility Complex (MHC)

The existence of white blood cell antigens independent of red blood cell systems was suggested in the 1950s following the detection of the first leukocyte antibody in a multi-transfused patient. This antibody reacted with white blood cells revealing an antigenic system on white blood cells termed the human leukocyte antigen (HLA) system (Van_Rood, 1969; Dausset and Colombani, 1970). In the early 1970s, an important role of the HLA system in graft rejection in humans was demonstrated through investigations in skin grafted and blood transfused patients (Ceppellini et al., 1971; Curtoni et al., 1972).

The HLA system codes for cell surface antigens and is located on the short arm of chromosome 6 within an estimated 3500-4000 KB of DNA. HLA-genes are divided into three classes, Class I, Class II, and Class III, based on molecular structure. Class I molecules in man include HLA-A, -B, and –C. These are synthesised and displayed by most of the cells of the body except cells that make up the central nervous system. Class II molecules in man include HLA-DR, -DQ and –DP. These are constitutively expressed on resting, non-activated macrophages and B-lymphocytes but can be up-regulated on most cells after IFNγ treatment. HLA Class II are involved in processing and presenting extra-cellular antigen to T-cells. Class III molecules include the C4, C2 and factor B components of the complement system (Trowsdale, 1997; Lehner and Trowsdale, 1998; Malcherek et al., 1998).

HLA antigens may be determined by serological techniques using lymphocytes as target cells, or by DNA techniques. Peripheral blood lymphocytes or separated T lymphocytes are used for Class I typing. B-lymphocytes are used for Class II typing, because DR and DQ antigens are constitutively expressed on B-cells, but not on resting T-cells. The antibodies to these antigens may be found in the sera of repeatedly transfused patients and in multiparious women. HLA antigens recognised as foreign on transplants leads to allograft rejection. HLA-Class II allo-types are also associated with autoimmune disorders, where the immune system has been abnormally activated to be

reactive against structures that it would normally tolerate (Altmann et al., 1990; Kikuti et al., 1997).

1.4. Blood transfusions

1.4.1. Introduction

Modern methods of blood collection, preservation, processing and storage have resulted in the wide availability of blood and blood components. It cannot be confirmed who conceived the idea of transfusing blood, but the earliest documented attempt to was during the 17th century (Spence et al., 1993). The first major contribution to blood transfusion science was when the A-B-O blood groups were discovered early in the last century. The next important step was the discovery of the Rh blood factor, in 1940. However, two world wars definitely accelerated the development of blood transfusion services (Greenwalt, 1997).

Blood transfusion is a valuable and vital therapeutic agent for improving oxygen supply to the tissues in surgical and non-surgical patients. The majority of blood administration in developing countries is in patients who are undergoing surgical operations. It is critical to improve haemoglobin levels in patients who have undergone surgery thereby decreasing the risk of death (Hebert et al., 1997).

1.4.2. Autologous blood transfusion

Autologous blood transfusion (ABT) is defined as transfusion of a patient's own blood. It is becoming increasingly popular as an alternative for allogeneic blood transfusion. The main purpose of autologous transfusion is to avoid the risk of infection associated with allogeneic blood such as hepatitis B, C, NANBNC, HIV, syphilis, malaria and other transfusion transmitted diseases. The safety of the allogeneic supply has been questioned since the first cases of transfusion transmitted human immunodeficiency virus (HIV) were reported in the early 1980s. The increasing awareness that allogeneic blood transfusion induces an immunomodulatory effect has also encouraged the use of autologous transfusion (Fiebig, 1998; Gillon and Thomas, 1999; Greenwalt, 1999).

Other reasons to shift from allogeneic to autologous transfusion are: shortfall in supply; alloimmunisation to red cells, platelets, leukocytes and plasma proteins; the risk of febrile, allergic and haemolytic reactions; graft versus host disease; clerical error; and finally cost-effectiveness (Kruskall et al., 1994; Dalen et al., 1996; Duffy and Tolley, 1997).

1.4.3. Autologous pre-deposit blood

Patients scheduled for elective surgery needing transfusion can be considered for autologous pre-deposit blood transfusion. The patient should be well motivated to complete the collection programme and be able to travel to the transfusion centre. This usually involves depositing up to four blood units within a relatively short interval. Pre-deposit blood is obtained at weekly intervals the last unit being collected a minimum of three days before operation. Blood is stored at 4^0 C and retransfused when needed during or after surgery. Collected blood can be stored for 30-36 days depending on the anticoagulant solution, and it should also be labelled and tested in the same way as allogeneic blood.

Candidates for pre-donation should be in as good health as normal blood donors. The criteria for pre-deposit autologous blood donation are:

a) Age between 17 and 70

b) Weight more than 50 kg

c) Good venous access in antecubital fossa

d) Both men and women should have a haemoglobin level of at least 110 g/l at referral

Blood is not to be taken from patients with the following criteria:

a) Significant cardiovascular disease

b) Uncontrolled hypertension (diastolic more than 100 mmHg)

c) Epilepsy

d) Haemoglobin level less than 110 g/l

e) Complicated pregnancy

f) Autonomic neuropathy

g) Active bacterial infection of any sort

h) Patients who are positive for anti HIV 1 and 2; anti-HCV; Hepatitis B surface antigen (HBs-Ag) or syphilis

i) Patients taking β-blockers (except if taken for anxiety or glaucoma and with a pulse rate above 60 beats/min) or other drugs that interfere with autonomic regulation

j) Patients age more than 70 years who have suffered a delayed faint after blood donation (Dzik, 1994a; Fiebig, 1998; Courouce et al., 1999).

Autologous pre-deposit blood transfusion cannot be used without supplementary allogeneic blood if more than five units are required. Pre-deposit transfusion can produce some of the same complications as regular allogeneic blood transfusion such as bruises at the joints and bacterial contamination (Gillon et al., 1999).

1.4.4. Autologous post-operatively salvaged blood

For first time in 1978, Schaff and his colleagues attempted to reinfuse the shed blood from mediastinal drainage during and after thoracic surgery. They showed a 50% reduction in the need for allogeneic blood (Schaff et al., 1978a; Schaff et al., 1978b; Schaff et al., 1979).

Patients undergoing total hip or knee replacement operations as well as cardiac surgery patients suffer significant blood loss at the site of surgery leading to the need to replace shed blood. This closed collection system is widely used for surgical procedures, such as cardiac, vascular, orthopaedic, urologic, trauma, gynaecologic and transplants surgery. The anticipated blood loss is 20 percent or more of the patient's estimated blood volume and there is no contamination of the area by bacteria or cancer cells. This procedure is generally not used in cancer surgery or surgery of the lower gastrointestinal tract (Clements et al., 1992; Elawad and Fredin, 1992; Dalrymple_Hay et al., 1999).

1.4.4.1. The method of blood collection in autologous post-operatively salvaged blood

Salvaged blood is collected from the surgical wound via a suction catheter, which has two lumens, one to deliver acid citrate dextrose (ACD), to act as both anticoagulant and preservative to the site of bleeding, and another to aspirate already anticoagulated blood. This is usually performed within the first 24 post-operative hours. Retrieved blood is collected for six hours and if the volume drained is at least 100 ml, reinfusion takes place. Collected blood is reinfused as soon as the drained volume reaches 500 ml, on the condition that it occurs within six hours after operation. Further reinfusion may be performed within the next six hours following the last reinfusion or after a further 500 ml has drained. This protocol may be repeated until 12 hours after operation or until a maximum of 1500 ml has been reinfused. The collected blood is washed or unwashed and re-infused through a 40-micron (μm) filter as in a standard blood-giving set (Clements et al., 1992; Elawad et al., 1992).

1.4.5. Allogeneic leukodepleted blood

Cellular blood components contain donor peripheral leukocytes and exposure to these may produce a number of transfusion complications. Clinical studies indicated that leukocyte-depletion decreased the risk of the transfusion complications associated with allogeneic transfusions. Leukodepletion is

practised in France, Ireland, Canada, Portugal and was recently introduced in the UK (Belardinelli et al., 1996; Georgeson, 1996; Sirchia and Rebulla, 1997)

Leukodepletion was officially implemented in the UK on the first of November 1998 and two months earlier at the Bristol blood transfusion centre. One of the most important reasons for leukodepletion was the presumed risk of transmitting new-variant Creutzfeldt-Jakob disease (nvCJD). The United States Food and Drug Administration (FDA) recommends withdrawal of components or products derived from donations by those at risk of infectivity for nvCJD. However, there is no report of any case of confirmed CJD among recipients of blood components, not even among recipients who received blood from donors who subsequently developed CJD (Heye et al., 1994; Beckman and Seghatchian, 1999; Seghatchian, 2000).

The main goals of universal leukodepletion are:

a) Prevention of febrile non-haemolytic transfusion reactions (FNHTRs)

b) Reducing graft rejection after haemopoietic stem cell transplantation, for example in multiply transfused, severe aplastic anaemia patients

c) Prevention of transmission of infectious agents such as cytomegalovirus (CMV), Human T-cell leukaemia/lymphoma virus (HTLV I/II), Human herpes virus 8 (HHV-8), Epstein-Barr virus (EBV), Trypanosoma cruzi and nvCJD

d) Intra-uterine transfusions, for all transfusions to infants below one year of age

e) To avoid transfusion-related acute lung injury causing the adult respiratory distress syndrome (ARDS). (Heddle and Blajchman, 1995; Ledent and Berlin, 1996; British Committee for Standards in Haematology, 1998; Larsson et al., 1998; Wadhwa et al., 2000).

FNHTRs are thought to occur as a result of several different mechanisms including: *1)* donor leukocytes recognised by the immune system of recipients via leukocyte antibodies as a consequence of HLA allo-immunisation; *2)* passive transfer of donor inflammatory cytokines produced by leukocytes during the period of storage and/or immune destruction of incompatible donor platelets by recipient antibodies (Slichter, 1998; Roddie et al., 2000).

ARDS is an infrequent but hazardous complication that may occur a few hours following blood transfusion. ARDS is characterised by symptoms of sudden respiratory distress, severe hypoxemia, fever and hypotention. The pathogenesis of transfusion-related acute lung injury causing ARDS is not fully understood. This syndrome is thought to be linked to the circulating antibodies produced against leukocytes leading to leukocyte aggregation and activation in the pulmonary micro-vasculature (Zupa„ska et al., 1999).

The current British standard defines that leukocyte-depleted blood components must contain less than 5×10^6 leukocytes per unit of red cells or per adult therapeutic dose of platelets. This should be achieved with at least 99 percent frequency and at least 95 percent statistical confidence. For achieving residual leukocyte counts of less than 5×10^6 per unit, leukocyte-depletion should be carried out under controlled conditions, ideally within 48 hours from the collection of the donor unit. The preparation of leukocyte-depleted blood components should be monitored under quality control guidance (Beckman and Seghatchian, 2000; Seghatchian et al., 2000).

1.4.5.1. Leukodepletion methods

Leukocytes can be removed from blood components by centrifugation, freeze/thawing and filtration with third generation filters. Filtration has progressed in three distinct stages. First-generation filters had the ability to remove large clots and particulate debris with a pore size of 170-240μm. Second-generation filters had a pore size of 40μm and were designed to reduce micro aggregates of fibrin, platelets and leukocytes by one $\log_{10}$. Third-generation filters remove free

leukocytes from blood, down to three log_{10} to a level less than 5×10^6 per unit (Norfolk and Williamson, 1995; Georgeson, 1996; British Committee for Standards in Haematology, 1998).

Leukodepletion can be carried out at the bedside during the transfusion or at the blood transfusion centre in the component-processing laboratory. Leukodepletion carried out a short time after blood collection has the advantage that release of cytokines or fragments of cell membrane is minimised (British Committee for Standards in Haematology, 1998; Roddie et al., 2000).

1.4.6. Allogeneic non-leukodepleted blood

A local volunteer donor pool provides allogeneic non-leukocyte-depleted blood for each blood transfusion centre. For safety the transfusion service should recruit and keep safe donors, and do high quality laboratory screening. In the United Kingdom, the risk of an infectious unit of blood being transfused is estimated at less than 1 in 2 million for HIV, less than 1 in 200,000 for HCV, and 1 in 50,000-200,000 for HBV. In the USA, this risk is estimated at less than 1 in 563,000-825,000 for HIV, 1 in 121,000 for HCV and 1 in 66,000 to 200,000 for HBV (Gillon and Thomas, 1999; Greenwalt, 1999).

In developing countries, allogeneic non leukocyte-depleted blood is used as packed red cells from which the derived plasma is derived and used for fractionation into blood components (Gillon and Thomas, 1999; Greenwalt, 1999). Allogeneic packed non- leukocyte-depleted blood has been widely used as an agent for improving oxygen supply to the tissues, especially in patients undergoing elective operations or in massive trauma cases. Some developing countries have shifted to implementing or recommending universal leukodepletion (Georgeson, 1996).

1.4.6.1. The allogeneic blood donation eligibility criteria

Blood donors with the following characteristics are eligible to donate blood. The blood donor must generally be at least 17 years of age, and a maximum of 75 years of age (although, some states in the USA permit younger people to donate with parental consent). However, most blood banks have no upper age limit. Donor weight should be at least 110 pounds (50 kg), and the donor must be in good health. The haemoglobin level should be at least 110 g/l. All donors must pass the physical and health history examinations given prior to donation. Volunteers with significant cardiovascular disease, uncontrolled hypertension, epilepsy, pregnancy, diabetes, active infection, and patients who are positive for anti HIV 1 and 2; anti-HCV; Hepatitis B surface antigen (HBs-Ag) or syphilis must not donate blood. Volunteers can donate platelets up to a maximum of four times per year, while whole blood can be donated once every eight weeks. The donor's body replenishes the fluid lost from donation within 24 hours. It may take up to two months to replace the lost red blood cells. The routine tests performed on drawn blood, are for ABO group (blood type), and Rh type (positive or negative), as well as for any unexpected red blood cell antibodies that may cause problems in the recipient. Some screening tests are also performed for evidence of donor infection with hepatitis viruses B and C, HIV 1 and 2 and syphilis. However, in some countries drawn blood is tested for human T-lymphotropic viruses (HTLV) I and II (Greenwalt, 1997; Fiebig, 1998; Prati et al., 1998).

1.5. Immunomodulation

Allogeneic blood transfusions, surgery and haemorrhage lead to suppression of the body's immune defences against pathogenic micro-organisms. These conditions invoke different biological mechanisms such that the impact on the immune system may be additive when two or more of these events occur together.

1.5.1. Surgery-induced immunomodulation

It is generally accepted that surgery-induced immunomodulation is manifest as increased post-operative infections such as pneumonia, peritonitis, urinary tract infections, septicaemia and wound infection (Baker et al., 1987; Cheadle et al., 1996).

Multiple trauma and surgically induced trauma are associated with depression of cell-mediated immunity associated with lymphocytopenia and impairment of T-cell proliferative response to mitogens. The decrease in proportion of CD4+ T-cells is greater than CD8+ T-cells (O_Mahony et al., 1984; Delogu et al., 2000)

In human and animal models, it was shown that during the early post-operative period the effects of surgical trauma on the immune system were decreased production of pro-inflammatory cytokines, and depressed in-vitro lymphocyte blastogenesis and proliferation (Kojima et al., 1983; Mack et al., 1996; Berguer et al., 1999).

Convincing evidence for the systemic effects of surgery-induced immunomodulation comes from controlled experiments using animal models. In a graft versus host disease (GVHD) model, skin incision to the F_1 murine host inflicted one day prior to injection of parental spleen cells produced impairment of popliteal lymph node enlargement. The spleen cells of F_1 mice displayed suppressor activity on local graft versus host (GVH) reactions when injected together with paternal cells into naive syngenic F_1 mice. This suppressor activity

was most marked 3 to 24 hours after the skin incision. Suppression was attributed to suppressor T lymphocytes in the host spleen and it showed that the local GVH reactions were suppressed by surgical trauma (Kojima et al., 1983).

In another animal model invasive surgery performed by laparotomy was associated with lower T-cell activation and more impairment in cell-mediated immunity than laparoscopic surgery with less tissue trauma (Berguer et al., 1998). Ben-Eliyahu et al investigated the effects of two stress paradigms, forced swim and abdominal surgery, on NK cell regulated tumour development in rat. They observed that in both stress paradigms, NK cell function was suppressed, and tumour development and metastasis were enhanced (Ben_Eliyahu et al., 1999a). Marshal et al observed a decrease in delayed hypersensitivity (DTH) response to a subcutaneous accumulation of Staphylococcus aureus and decrease in phagocytic clearance of Escherichia coli by hepatic macrophages following surgical trauma (Marshall et al., 1987).

Surgery-induced immunomodulation varies according to the severity of the surgery and patient characteristics. For example cholesystectomy and rectosigmoid carcinoma resection performed by laparoscopic surgery results in less immunomodulation than the same operation performed by conventional open surgery involving laparotomy (Berguer et al., 1999).

The general impression is that major surgery suppresses the ability of T-cells to produce pro-inflammatory cytokines without initially enhancing producing of anti-inflammatory cytokines (Berguer et al., 1999). Indeed levels of IL-4, one of the index anti-inflammatory cytokines, was depressed in some studies (Baker et al., 1987; Berguer et al., 1999). Later, as the immune system recovers an anti-inflammatory profile emerges with sustained increases in IL-10 production. In patients with colonic cancer undergoing recto-sigmoidal surgery by laparotomy levels of IL-1β and IL-6 were elevated compared to control patients operated by laparoscopy (Berguer et al., 1999; Berguer et al., 2000). In a similar study of cholesystectomy IL-2, IFNγ and TNFα were all lower after open surgery than after laparoscopic surgery. IL-2 levels only rose transiently or not at all. IL-10 levels

were initially unaffected, but rose later, supporting the view that an anti-inflammatory profile emerged later after surgery, but monocyte antigen presenting function appeared to be unaffected (Hensler et al., 1997).

It was suggested that PGE_2 released by macrophages may have been both the source of suppression of the inflammatory response leading to decreased IL-2 and IFNγ activity and the cause of the deviation to an anti-inflammatory profile (Droller et al., 1979; Duffie et al., 1988; Eisengart et al., 2000).

Interestingly the anti-inflammatory pathway of T-cell differentiation (Th_2) was associated with increased B-cell help (Di_Padova et al., 1988). This may explain the odd observation that polyclonal B-lymphoblastoid activation occurred between five and 14 days after cholecystectomy (Di_Padova and Durig, 1988).

The local sites of trauma appear to be rich in IL-10 despite normal IL-10 blood levels. The immunosuppressive role of IL-10 is well known; it inhibits the synthesis of anti-inflammatory cytokines and antigen-presenting function of monocyte/macrophages by down-regulating expression of MHC Class II, CD54, CD80 and CD86, preventing the release of oxygen free radicals and nitrogen intermediates. Increased systemic IL-10 levels were related to the severity of injury and to post-traumatic complications (Klava et al., 1997; Koller et al., 1998).

By contrast, after cardiac and aorta-femoral by-pass vascular surgery the picture was different with elevated TNFα, IL-6 and C-reactive protein (CRP) level, but this was related to volume of blood lost and may be more a manifestation of haemorrhage-induced immunomodulation (see section 1.5.2), than surgery-induced immunomodulation (Gadaleta et al., 1994; Hattler et al., 1995; Diegeler et al., 2000).

Immunomodulation-induced by major surgery and allogeneic blood transfusion appear to be additive on the immune system (see section 1.5.3). In a cohort study, NK cell function, IL-2 and IFNγ production were investigated in

136 patients who underwent surgery for gastrointestinal malignancy. The results showed a significant decrease in NK cell function, IL-2 and IFNγ production after operation in both the transfused and non-transfused groups. However, the proportion of post-operative infections in transfused patients was 28% compared with 5% in the non-transfused patients. Thus, extra deleterious effects on cellular immunity resulted from transfusion (Quintiliani et al., 1997).

1.5.1.1. Effect of anaesthesia and morphine on surgery-induce immunomodulation

General anaesthesia per-se is associated with transient in-vitro changes, but there is little if any evidence to support the view that it causes overt or lasting symptoms of immunosuppression. For example, in one study basal NK cell cytotoxic function against K562 was significantly depressed after general anaesthesia alone compared to surgery plus anaesthesia. This depressed NK cell cytotoxicity was restored to pre-operative levels by treating NK cells with IFNα (Kutza et al., 1997).

However, hypothermia, a common surgical complication under thiopental general anaesthesia, was shown in an animal experiments that it was associated with decreased NK cell function and compromised host resistance to metastatic tumour spread. This was attributed to adrenergic mechanisms (Ben_Eliyahu et al., 1999a; Ben_Eliyahu et al., 1999b).

In Kutza study has been proposed that a decrease in NK cell activity after surgery and anaesthesia may lead to enhanced susceptibility to infection and tumour dissemination (Kutza et al., 1997; Ben_Eliyahu et al., 1999a).

In breast cancer patients who underwent surgery, NK cell function and lymphoproliferative response to phytohaemagglutinin (PHA) were reduced. No evidence was found to suggest this effect be related to a particular anaesthetic regime (Stanojevi‡_Baki‡ et al., 1999).

The opioid antagonist naltrexone appears to reverse the deleterious effects of surgery in the rat. The effect on surgically induced immunomodulation was evidenced by reversal of the decreased NK cell cytotoxic function, reduced B-cell and T-cell proliferation, and decreased IFNγ production. Naltrexone administered at the time of surgery and every 4 hours thereafter reversed these effect (Nelson et al., 2000)

Exogenous morphine may induce down-regulation of lymphocyte, granulocyte and macrophage activity. In patients who underwent abdominal surgery for uterine carcinoma a decrease in NK cell function and lymphoproliferative response to PHA, was observed. Patients treated with 10 mg of intra-muscular of morphine immediately before and after operation had a lower response to PHA two hours after the operation than those treated with 100 mg of another opioid derivative, tramadol (Sacerdote et al., 2000)

The effect of exogenous morphine on NK cell activity was studied in patients who underwent hysterectomy. These patients were treated with 0.5mg morphine administrated intrathecally. NK function was decreased after surgery (Yokota et al., 2000). In a study on sixteen patients underwent coronary artery by-pass grafting an increase in endogenous morphine was observed that it may induce immunomodulation after surgery (Brix_Christensen et al., 1997).

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1.5.2. Haemorrhage-induced immunomodulation

Clinical manifestations of haemorrhage-induced immunomodulation are rarely described except in its most extreme form of multi-organ failure because blood loss is seldom sustained for long periods without correction. Furthermore, haemorrhage-induced immunomodulation may often be masked by the immunomodulatory effects of surgery. In experimental rodent models substantial evidence has been obtained for systemic immunomodulation following severe uncorrected blood loss. In one of these experiments a significant increase in circulating transforming growth factor-beta (TGF-β) up to 24h following haemorrhage, was observed. The elevated TGF-β plasma level was associated with a decrease in concanavalin A-induced splenocyte proliferation and antigen presentation by splenic macrophages (Ertel et al., 1992b; Ayala et al., 1993; Ayala et al., 1997).

In another animal experiment a decrease in splenocyte T-cell proliferation was associated with an increased in IL-4 and IL-10 synthesis. This IL-4 and IL-10 increase was preventable by addition of ibuprofen as COX-2 inhibitor or monoclonal antibody against TGF-β. The TGF-β is a growth factor involved in tissue repair and T-cell impairment (Ayala et al., 1994).

In experimental models, mitogen induced proliferative responses by splenic lymphocytes were decreased, as was antigen presenting function by peritoneal macrophages and hepatic Kupffer cells. Expression of MHC Class II antigens by Kupffer cells and macrophages was suppressed. At the macrophage cell surface there was less expression of MHC Class II and IL-2R following haemorrhage (Abraham and Freitas, 1989; Abraham and Chang, 1992; Chaudry et al., 1995). Zhu et al observed an increase in TNFα, IL-6 and TGF-β levels in the plasma and an increase in TNFα, IL-1β and TGF-β mRNA message in Kuppfer cells, but not splenic macrophages (Zhu et al., 1995). In a murine model, Ertel et al demonstrated an increased release of PGE_2 by macrophages and Kupffer cells. Chloroquine treatment inhibited the secretion of PGE_2 by

macrophages leading to decreased mortality from sepsis mice after haemorrhage compared to controls (Ertel et al., 1991; Ertel et al., 1992a).

In a rabbit model of haemorrhage-induced immunomodulation were prevented by prior treatment with polyunsaturated fatty acids that suppressed PGE_2. In this study, the potential role of PGE_2 in the development of multiple organ failure and the effects of antiserum directed against lipopolysaccharide (LPS) as the inducer of arachidonic acid metabolite, were investigated. Antiserum to LPS given intravenously at the onset of haemorrhage and four hours after resuscitation lead to no increase in the circulating PGE_2 level after haemorrhage up to 24 hour after resuscitation. Lower LPS concentrations and significantly higher survival rates were observed after administration of LPS antiserum (Yao et al., 1996).

COX-2 converts arachidonic acid to PGE_2 that is further metabolised to various prostaglandins, prostacyclin and thromboxane A_2. COX-2 inhibitors, such as ibuprofen suppress nuclear factor kappa B (NF-kB) dependent synthesis of PGE_2 in macrophages. In turn PGE_2 abrogates synthesis of TNFα and IL-6. In mouse macrophages were exposed to Escherichia coli LPS in the presence of ibuprofen. COX-2 mRNA expression and PGE_2 was inhibited by ibuprofen. These agents attenuated LPS-induced NF-kB activity. (Gadd and Hansbrough, 1990; Mansilla_Rosello et al., 1996; Lo et al., 1998; Wissink et al., 1998).

In a more extreme model of haemorrhage-induced immunomodulation leading to immunosuppression and multi-organ failure following haemorrhagic shock, serum PGE_2 levels were reduced by antiserum directed against LPS (Manning et al., 1989; Ayala et al., 1997; Asanuma et al., 1999).

In a mouse model of haemorrhage-induced immunomodulation anti-IL-10, anti-IL-4 and ibuprofen inhibited PGE_2 augmented IL-10 production by T-cells and reversed the in-vitro suppression of T-cell proliferation (Napolitano et al., 1996; Ayala et al., 1997). It has also been reported that a significant decrease in IL-3, IL-5 and IL-6 synthesis occurred after haemorrhage that may lead to

impaired recruitment and maturation of monocyte/macrophage and antibody producing B-cells (Abraham and Freitas, 1989; Meldrum et al., 1991).

The effects of experimental haemorrhage-induced immunomodulation were also reversed by treatment with IFNγ, or antibody to TGF-β or platelet agglutinating factor (PAF) antagonists (Stewart et al., 1995; Zellweger et al., 1995).

Clinical changes suggestive of haemorrhage-induced immunomodulation have been observed in colorectal cancer patients after being bled for 'pre-deposit' blood transfusions, especially after the donation of two blood units, a sustained decrease in NK cell function was observed in-vitro (Marquet et al., 1993b).

Lasek et al demonstrated for the first time a decrease in NK cell function, associated with blood donation by healthy donors (Lasek et al., 1987). Then Ford et al confirmed this observation (Ford et al., 1987).

Marquet et al described a decrease in NK cell function at least five days after blood donation by healthy donors. However, this alteration was not associated with adverse clinical manifestations (Marquet et al., 1993a; Mathiesen et al., 1994; Quintiliani et al., 1997).

It is assumed that haemorrhage is a potent stimulator of corticosteroid and prostaglandin secretion, both of which inhibit NK cell function. Another possibility, which has been observed consequence of blood donation is that haemorrhage may lead to a shift of immature NK cells from the bone marrow into the blood leading to the recruitment of immature NK cells with lower activity in blood circulation (Marquet et al., 1993a; Marquet et al., 1993b; Rhind et al., 1999).

In a clinical study following burning, decreases in the absolute number of circulating lymphocytes, CD4+ cells and the ratio of CD4+ to CD8+ were observed. There was decreased expression of monocyte HLA-DR, lymphocyte

proliferation after mitogen stimulation, CD4+, CD8+, IL-2R and transferrin receptor after mitogen stimulation (Hansbrough et al., 1984; Zapata_Sirvent and Hansbrough, 1993).

1.5.3. Allogeneic blood transfusion-induced immunomodulation

The term blood transfusion-induced immunomodulation is generally used to refer to down regulation of the human immune system in response to allogeneic blood transfusions. Clinical evidence for allogeneic blood transfusion-induced immunomodulation is well documented (Blumberg and Heal, 1996b).

In 1945, Medawar noticed that some transfused animals rejected skin grafts from the blood donor in as short a time as animals immunised by prior skin grafts. This 'second-set' phenomenon occurred only if donor antigens shared by donor blood and skin were capable of inducing cell-mediated immunity. He concluded that tissues expressed the same antigens as blood and this information strongly influenced the early years of human organ transplantation (Brent and Medawar, 1967; Lance and Medawar, 1969). Medawar and others demonstrated that the administration of intravenous alloantigen could be tolerogenic and modulate the immune system against an allograft, although this phenomenon was not seriously addressed in a clinical trail until the end of 1960s (Billingham et al., 1969; Brunson and Alexander, 1990; Perkins et al., 1996).

1.5.3.1. The beneficial effects of allogeneic blood transfusion

In the early 1970s, Opelz reported that allogeneic blood transfusion improved the outcome of renal allografts. He observed that when allogeneic blood transfusion was regularly administered during dialysis, the probability of graft survival was increased in kidney transplant recipients. It was shown that allogeneic blood transfusion was associated with immunomodulation in recipients, thus pre-transplant allogeneic transfusion became routine policy in kidney transplantation centres during the 1970s and the early 1980s (Opelz et al.,

1973; Rashid et al., 1975; Rashid and Sengar, 1978; Sengar and Rashid, 1978; Opelz et al., 1997).

Opelz and Terasaki further demonstrated that the patients who received whole blood had the best allograft outcome compared to those who received other blood components. The best blood component after whole blood was packed cells. Those who received leukodepleted blood including frozen/thawed or washed red blood cells (RBC) had outcomes no better than non-transfused patients (Opelz and Terasaki, 1976; Opelz and Terasaki, 1978).

Until this time allogeneic transfusions were only thought to induce RBC allo-antibody leading to haemolytic and non-haemolytic transfusion reactions. Billingham and Chaplain, in the early 60s, were among the first to recognise a role for leukocytes and platelets in precipitating febrile reactions after blood transfusion, and Perkins demonstrated a semi-quantitative relationship between non-haemolytic febrile reactions and contaminating leukocytes, particularly granulocytes (Perkins et al., 1996). Thus transfusions appeared to have two contrasting effects on allo-immunity; they stimulated as humoral immunity inflammatory reactions, but suppressed cellular immunity to HLA antigens.

Auto-immune processes also appear to benefit from allogeneic transfusion-induced immunomodulation since transfusions given to patients with Crohn's disease modified the course of disease and those who received transfusions during bowel resection operations had less post-transplant recurrence of their disease than non transfused patients (Peters et al., 1989; Williams and Hughes, 1989; Tartter, 1995). Allogeneic leukocyte transfusions may be beneficial in women suffering primary recurrent miscarriage. Higher successful pregnancies have been claimed in the women who received paternal cells compared to those who received their own cells (Mowbray et al., 1987; Mowbray, 1988). This however, remains controversial.

1.5.3.2. The detrimental effects of allogeneic blood transfusion

During the last three decades, awareness of the potential risks associated with blood components due to impairment of immunologic function in recipients, particularly those associated with cell mediated immunity, has increased (Blumberg and Heal, 1996b; Dzik et al., 1996; Blajchman, 1998).

The functional, cellular and molecular changes that accompany allogeneic blood transfusion-induced immunomodulation have been studied extensively in rodent models and in man. Experiments in animal models supported the idea that allogeneic transfusion alters the immune system by enhancing production of anti-inflammatory cytokines, decreasing NK cell number and NK cell cytotoxicity and decreasing macrophage migration in response to inflammatory stimuli. In man, the effects of allogeneic transfusion include: decreased CD4:CD8 lymphocyte ratios; decreased NK cell function; decreased antigen presentation; suppression of lymphocyte blastogenesis; and reduced delayed hypersensitivity (Bordin et al., 1994; Vamvakas et al., 1995; Blumberg and Heal, 1998).

These alterations are linked to enhanced metastatic spread and growth of tumour cells (Francis and Shenton, 1981; Horimi et al., 1983; Tartter and Francis, 1988; Busch et al., 1993a; Hoynck_van_Papendrecht et al., 1993). Animal models have shown that allogeneic transfusion the possibility after allogeneic blood transfusion is an independent risk factor of enhanced tumour recurrence (Gantt, 1981).

Retrospective and prospective clinical studies supported the idea that allogeneic transfusion had detrimental effects on host immune defences. Burrows and Tarter were the first to show a deleterious effect of allogeneic transfusion in patients with colorectal cancer (Burrows and Tartter, 1982). They and others reported that administration of allogeneic blood was an important factor in recurrence of colorectal malignancy and decreased survival. Allogeneically transfused patients also displayed higher rates of post-operative infection than non-transfused patients did (Blumberg and Heal, 1987; Burrows et

al., 1987; Tartter, 1988c; Tartter, 1988a; Jensen et al., 1992; Busch et al., 1993b; Houbiers, 1994). Similar results were observed in patients undergoing radical resection for oesophageal carcinoma and gastric adenocarcinoma. In both studies allogeneic transfusion was associated with poor outcome and decreased survival (Fong et al., 1994; Dresner et al., 2000).

Furthermore, in another study on patients who underwent elective colorectal surgery a correlation between the development of post-operative infection and allogeneic transfusion was observed despite antibiotic prophylaxis (Jensen et al., 1990).

Leukocyte-depleted allogeneic RBC or buffy coat depleted packed RBC are also associated with higher infection rates and lower survival rates compared with non-transfused patients (Houbiers et al., 1994a; Mathiesen et al., 1998; Innerhofer et al., 1999a; Innerhofer et al., 1999b).

In patients with non-malignant diseases who received spinal fusion surgery the number of units transfused was the major predictor of increased in-hospital infection, as well as days on antibiotics and length of stay (Triulzi et al., 1992).

Studies on orthopaedic patients including joint replacement have demonstrated that the incidence of post-operative infections and delayed wound healing after surgery in patients receiving allogeneic transfusions was higher compared to those receiving autologous or no transfusions (Murphy et al., 1991; Fernandez et al., 1992; Triulzi et al., 1992; Howard et al., 1993; Carson et al., 1999; Innerhofer et al., 1999b).

Moreover, cytokine studies in patients undergoing hip replacement surgery who received allogeneic transfusions showed no change in IL-2 but IL-10 and IL-4 production were enhanced (Kirkley et al., 1998). Cytokine studies in patients undergoing recto-sigmoidectomy who received allogeneic transfusions showed an increment in blood levels of IL-6 (Ishijima and Suzuki, 1998).

Evidence from animal models further supports the idea that allogeneic transfusion is associated with more bacterial infections. The percentage of viable bacteria in some organs including liver, lung, spleen, lymph nodes, peritoneal cavity and blood was significantly higher in allogeneic transfused compared to non-transfused groups. (Tartter, 1989; Scorza et al., 1990; Gianotti et al., 1992; Eaves_Pyles and Alexander, 1997). Also, transfused mice showed higher mortality rates compared to non-transfused mice (Tartter, 1995; Blajchman, 1998).

In addition, experiments in animal models showed an increase IL-4 levels and a decrease in IFNγ levels after allogeneic blood transfusion (Babcock and Alexander, 1996; Frede et al., 1997; Levy et al., 1997).

It was shown that a significant increase in PGE_2 synthesis after allogeneic blood transfusion in an animal model may lead to impair of cell-mediated immunity. This deleterious effect was abrogated by indomethacin (Shelby, 1987; Shelby et al., 1987; Perez et al., 1997).

At the pragmatic level the detrimental effects of allogeneic transfusions can be avoided by reinfusion of autologous blood drained from the operation site (Schaff et al., 1978a; Schaff et al., 1979). Results from patients who received salvaged autologous blood showed that this approach prevented detrimental effects on cell mediated immunity in recipients compared with allogeneic transfusions (Murphy et al., 1991; Heddle et al., 1992; Mezrow et al., 1992; Heiss et al., 1993; Houbiers et al., 1994a; Heiss et al., 1997a).

In two studies of patients who received autologous post-operative unwashed salvaged blood, the concentration of released cytokine, including IL-6 and IL-8 in transfusate, was not reduced by filtration. This lead to an increase IL-6 and IL-8 in concentration of these products in the recipients' plasma, whereas in those patients who received washed and centrifuged shed blood, no elevation in released cytokines was observed in the recipients' plasma. Similar, increases

in plasma concentrations of IL-6 were shown in patients who received post-operatively salvaged blood (Arnestad et al., 1994; Arnestad et al., 1995).

Autologous blood salvage reduces the need for allogeneic blood whereas some results demonstrated that the number of patients who needed allogeneic blood transfusion was reduced up to 50% in Schaff's study and 45% in Dalen's study (Schaff et al., 1978a; Dalen et al., 1996). Furthermore, it is cost effective and the alkaline pH of salvaged blood reduces acidosis associated with massive transfusion (Ritter et al., 1994; Spence et al., 1995; Dalen et al., 1996; Blumberg, 1997; Duffy and Tolley, 1997).

1.6. Mechanisms of immunomodulation-induced by surgical trauma

1. IL-10. IL-10 may cause immunomodulation. Monocyte HLA-DR, mRNA expression and serum IL-10 levels were studied in 48 patients who underwent major elective resectional surgery. There was a significant decrease in monocyte HLA-DR expression 24 hours after surgery that remained low during the first postoperative week. An increase in IL-10 mRNA expression and serum IL-10 were also observed on the first postoperative day. Furthermore, there was a significant correlation between HLA-DR antigen expression and the presence of IL-10 mRNA transcript on the first post-operative day (Klava et al., 1997).

The contribution of polymorphonuclear neutrophil fractions to the development of surgery induced immunomodulation was evaluated. The concentration of IL-10 in culture supernatants of 30 individual PMN fractions was higher in traumatised patients compared with healthy donors (Koller et al., 1998).

In one clinical study it has been proposed that the dominance of anti-inflammatory reaction may cause surgery-induced immunomodulation (Decker et al., 1996).

In an animal model it was shown that severe injury leads to a shift in production of anti-inflammatory cytokines including IL-4, IL-5, IL-6 and IL-10, which may contribute to immunomodulation (Mack et al., 1996).

In another animal experiments was shown that bilateral femur fractures and mechanical tissue injury, lead to decreased splenic T-cell proliferation in response to concanavalin-A (con-A). Furthermore, there is a decrease in IL-2 and IFNγ synthesis in cultures of con-A stimulated splenic T-cell. Interestingly, IL-10 synthesis was decreased in these traumatised rats (Meert et al., 1998).

2. PGE_2. A role for PGE_2-mediated suppression and high production of the arachidonic acid metabolite PGE has also been proposed. One group showed that the PGE derivative impaired neutrophil migration into burn wounds.

It also decreased macrophage migration in response to inflammatory stimuli, decreased NK cytotoxic function and down-regulated expression of cell-surface molecules involved in antigen presentation. This increased the risk of development of MOF(Waymack et al., 1987; Winslow et al., 1996; Koga et al., 2000; Liu et al., 2000b).

3. Reactive oxygen species (ROS). In a clinical study a significant decrease in the in-vitro NK cytotoxic function following trauma was unaffected by the addition of anti-IL-4, anti-TGF-β1 and anti-IL-10 antibodies. But, removal of monocytes from buffy coat mononuclear cells reversed this NK cell suppression suggesting that immunomodulation may have been attributed to production of reactive oxygen species (ROS) by monocytes (Joshi et al., 1998).

1.7. Mechanism of immunomodulation induced by allogeneic blood transfusion

Transfused allogeneic blood, and its various components, may interact with the immune system in a variety of ways. Several hypotheses have been suggested and each of them has been experimentally investigated. They are briefly explained below. However, the precise mechanism of immunomodulation induced by allogeneic blood transfusion has not yet been elucidated.

1. Role of leukocytes (WBC). Some publications investigated the role of allogeneic WBC. They proposed that donor allogeneic leukocytes express mismatched HLA Class II antigens that react with recipient T-cells by increasing reactivity of the recipient's cytotoxic T-cells. The subsequent interaction between activated recipient cytotoxic T-cells and the blood donors' mismatched HLA Class II antigens leads to proliferation and differentiation of T-cells that down-regulate cell-mediated immunity in the recipient (Dzik, 1994b; Blumberg and Heal, 1996a; Blajchman, 1998; Vamvakas and Carven, 1998b; Vamvakas and Blajchman, 2000).

However, the results obtained with buffy coat-depleted blood components do not support the view that donor WBCs are the only cause of immunomodulation, since this appears to result at least in part from the allogeneic erythrocyte transfusion (Houbiers et al., 1994a; Andersen et al., 1998; Kirkley et al., 1998; Mathiesen et al., 1998; Innerhofer et al., 1999a; Innerhofer et al., 1999b).

In recent rat study transfusion of leukocyte-depleted blood impaired intestinal anastomotic healing. Both allogeneic leukocyte-depleted blood and non-leukocyte-depleted blood were associated with an increase in anastomotic abscesses compared with the "no transfusion" group (Apostolidis et al., 2000).

2. Role of red blood cells (RBCs). Tarnvik and others showed that the response of human peripheral blood lymphocytes to mitogen was equally

enhanced by autologous and allogeneic erythrocytes (Tarnvik, 1970; Shau and Golub, 1988). In-vitro studies following a single blood transfusion demonstrated how RBCs increases T-cells suppressor numbers and function in response to Con-A and PHA and how monocytes increased the production of PGE_2 (Gafter et al., 1992).

Kalechman et al hypothesised that peripheral blood lymphocytes have receptors for autologous RBC that are barely expressed in the resting cells but become evident after mitogenic stimulation. They showed that T-cell proliferation in response to mitogens, production of TNFα, IFNγ, IL-2, IL-6, and IL-2R expression on MNC were increased in the presence of autologous RBC (Kalechman et al., 1993)

3. Effects of storage. A series of investigations by Mincheff et al showed that blood storage contributes to the alteration of the recipients' immune system. In these studies, the role of leukocyte necrosis during refrigerated blood stored up to 2 weeks was studied. Production of IgM and IgG_1 anti-donor antibody was increased as a consequence of transfusion of lysed donor leukocytes. In addition, transfusion of lysed donor leukocytes led to suppression of subsequent delayed hypersensitivity (DTH) to donor antigens, possibly through induction of anergy or apoptosis in allo-reactive recipient cells (Mincheff et al., 1993; Mincheff et al., 1995; Mincheff and Meryman, 1995).

In a study by Mynster and et al, the leukocytes of heparinised blood were collected from whole blood and SAGM (saline, adenine, glucose and mannitol) blood after 1, 21 and 35 days. These were stimulated and cultured by LPS and PHA to measure TNFα and IL-2 synthesis after PHA stimulation. IL-2 and TNFα synthesis decreased in both stored whole blood and SAGM blood compared to controls. After PHA stimulation TNFα synthesis was decreased and IL-2 was undetectable in LPS-stimulated cultures but the synthesis of both cytokines was dependent on storage time. Thus, transfusion-induced immunomodulation blood storage may change with in storage time (Mynster et al., 1998).

Zallen et al described how stored packed red cells can provoke neutrophils to release inflammatory cytokines including IL-8, IL-1, TNFα and secretory phospholipase A_2 possibly leading to multiple organ failure after transfusion (Zallen et al., 2000).

In another study, increased levels of anaphylatoxins in whole blood and plasma were observed in refrigerated blood. Concentrations of the anaphylatoxins C3a and C5a increased during storage. The transfusion of large amounts of these activated complement components may increase the risk of developing organ dysfunction (Hyllner et al., 1997).

4. Other factors. In patients haemodialysed with cuprophane membranes, the number of NK cells and their cytotoxic activity against K562 targets was significantly decreased, compared to patients haemodialysed with polyacrylonitrile membranes. This reduced NK function could be reversed by changing membranes from cuprophane to more biocompatible membranes (Cala et al., 1990; Gascon et al., 1996).

In 1992, Riches et al showed that the anticoagulant lithium heparin induced IL-6 and IL-1 production in stored whole blood, whereas EDTA was ineffective (Riches et al., 1992).

In a randomised clinical trial comprising 697 transfused patients who underwent colorectal cancer surgery, plasma micro aggregates and citrate were insignificant as risk factors in post-operative infection (Houbiers et al., 1997).

In a review of patients who underwent elective surgery for gastric cancer a large quantity transfused blood (>1500 ml), that took longer to administer was associated with a worse clinical outcome (Bellantone et al., 1998).

Some publications suggested that soluble HLA Class I, CD95 (Fas) and Fas ligand (FasL) molecules are present in blood components were biologically active in-vitro and may produce immunomodulation. These molecules, if present

in sufficient concentration, could inhibit mixed lymphocyte responses and cytotoxic T-cell activity in allogeneic and autologous combinations and induce apoptosis in CD95+ cells. Also, these molecules were able to down-regulate NK cell function via a receptor for Class I molecules (Ghio et al., 2000; Puppo et al., 2001).

Kirkley demonstrated an increase in anti-inflammatory cytokine synthesis in patients receiving allogeneic blood transfusion compared to those who received autologous blood. He proposed that shifting toward anti-inflammatory reaction may be attributed to allogeneic blood transfusion-induced immunomodulation (Kirkley et al., 1998).

In animal and human models, it was also shown an increased PGE_2 production in allogeneic transfused patients or animals (Ross et al., 1990; Gafter et al., 1992; Perez et al., 1998; Koga et al., 2000).

1.8. Goals of this study

1- Develop an in-vitro method for quantitating the absolute number of natural killer precursor (NKp) cells in the peripheral blood mononuclear cells.

2- Measure the frequency of NKp cells before and after surgery and transfusions with:

a) allogeneic or autologous blood

b) allogeneic leukodepleted or allogeneic non-leukodepleted blood

c) non-transfused patients

3- Measure in-vitro synthesis of IFNγ, IL-10 and IL-4 before and after surgery and transfusions.

4- Investigate the correlation between immune system changes after major surgery and blood transfusions.

5- Investigate whether CD56 is an adequate marker for measuring functionally active NK cells that lyse the K562 target cell line.

6- Investigate the correlation between post-operative infections and the type of blood transfusion.

7- Investigate the correlation between post-operative infections and NKpf changes.

Section 2.

Materials and methods

2.1. Study patients

2.1.1. Ethical approval

The experiments described in this study were fully approved by the local hospital research ethics committee and informed consent was obtained from all volunteers. Patients were interviewed and informed about this study. If they agreed to take part, the voluntary consent was signed.

2.1.2. Patient criteria

All patients studied underwent elective total knee replacement (TKR) or total hip replacement (THR) surgery at the Avon Orthopaedic Centre, Southmead hospital, Bristol. Patients were excluded from the study if they had pre-existing infection, previous blood transfusion, malignancy, autoimmune disorders and diabetes.

2.1.3. Sample collection

Heparinised blood was collected from 125 patients who voluntarily took part in this study. The PBMC and plasma were separated later. The pre-operative samples were collected at the orthopaedic assessment clinic, Avon Orthopaedic Centre, Southmead Hospital. The post-operative samples were taken five days after operation on the wards. The blood samples of 29 patients who had signed the voluntary consent were not collected for the following reasons; seventeen cases for poor veins; five cases for diabetes; four cases for recent blood transfusion history. Samples from two patients were taken in the wrong anti-coagulant and one had cardiac problems that prevented the operation going ahead.

Seventeen post-operative samples were not collected, for different reasons: nine patients were discharged earlier than expected (before five days); two samples were not taken; four patients refused to be bled; one sample was lost and one patient was transferred to I.T.U following cardiac arrest during his operation. Twelve patients were treated at a hospital other than the Avon Orthopaedic Centre where the pre-operative samples had been collected. There were five paired collected samples that were excluded from the study due to diabetes (2), recent blood transfusions (2) and pre-existing infection (1).

2.1.4. Clinical Data

Data collected from patients' medical records included: age; gender; admission haematocrit (Hct); white blood cell count; total absolute lymphocyte count before and after operation; surgical procedure; type of operation; diabetes history; length of hospital stay; days with fever more than 38^0C; number and type of blood transfusions including post operative autologous blood collection; and postoperative infection including culture result and site of infection.

2.1.5. Patient groups and numbers

120 Patients were divided into five groups (Gp) according to the type of blood transfusion:

- Gp-1: Allogeneic non-leukodepleted blood, 8 patients
- Gp-2: Allogeneic leukodepleted blood, 30 patients
- Gp-3: Autologous post-operatively salvaged blood, 40 patients
- Gp-4: Autologous pre-donated blood, 10 patients
- Gp-5: No blood transfusion, 32 patients

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The Neubauer counting chamber

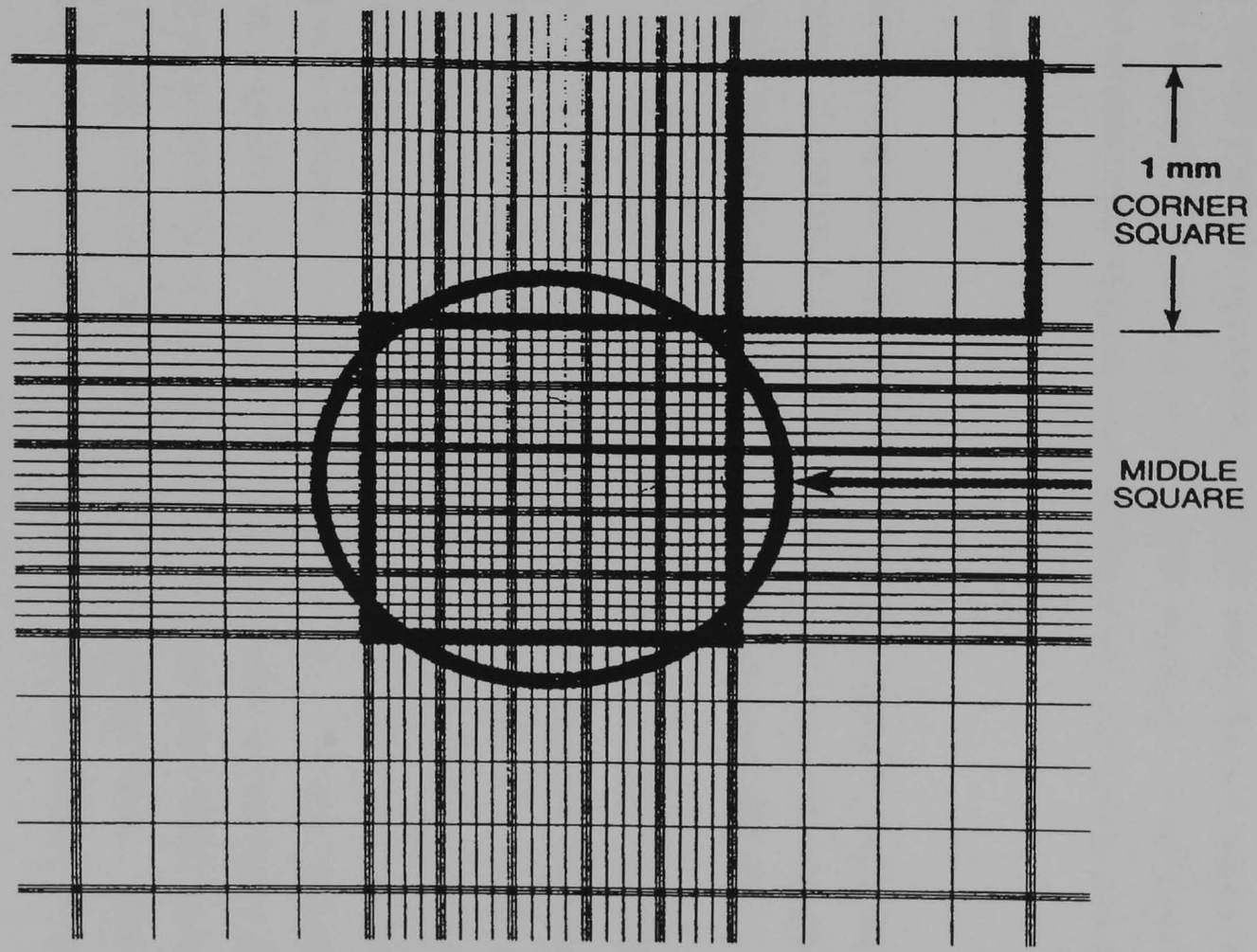


Figure-6.2: Schematic diagram of the squares of the Neubauer counting chamber. The centre was used for counting PBMC. The four corner squares of chamber were used for counting when the number of PBMC in the centre square was below 100.

2.4. Cell freezing in Dimethysulphoxide (DMSO)

2.4.1. Material

20% Dimethyl sulfoxide (DMSO) medium

20% DMSO medium consisting of RPMI 1640 (Sigma) supplemented with 20% DMSO (Sigma) was stored at 4^0C until required. Antibiotics containing penicillin and streptomycin (Sigma) were added prevent bacterial contamination.

2.4.2. Method

For preserving the viability of PBMC for future assays, cells were stored in liquid nitrogen. To prevent ice crystal damage to cells and maintain a viable state, cells were frozen in a mixture of fetal calf serum and DMSO.

The cell suspension was diluted in CCM-1 containing 50% FCS to concentration of 5 x 10^6 cells per ml and placed on crushed ice to cool. The 20% DMSO medium was added drop-wise to an equal volume of the cell suspension giving a final concentration of 20% FCS and 10% DMSO medium. The cell suspension was dispensed into pre-cooled cryovials by putting them in crushed ice with between 5-15 x 10^6 PBMC per vial. All the procedures were carried out in a laminar flow hood. The cryovials were immediately placed in a freezing tray of a small liquid nitrogen container because DMSO is too toxic for cells using a 'plug' inserted into the neck of the Dewer flask. The cells were gradually frozen in the nitrogen vapour at a rate of approximately 1^0C per minute.

Cryovials were left in the small liquid nitrogen container for a minimum of two hours before being transferred to racks and stored in the liquid phase of a large liquid nitrogen tank at -190^0C.

2.5. Thawing of cryopresevered cells

2.5.1. Material

Thawing medium

Thawing medium consisting of RPMI 1640 (Sigma) supplemented with 2% fetal calf serum (Sigma) and 3mM L-glutamine (Sigma) was stored at 4^0C until required.

2.5.2. Method

Frozen cells were thawed carefully to prevent cell loss and preserve viability for functional assays. The comparison between frozen/thawed PBMC and fresh PBMC for NK cell functional assay in a healthy adult sample (AG) were shown to be comparable (see section 4.2.5).

Cryovials were removed from the liquid nitrogen tank and immediately thawed in a pre-warmed water bath at 37^0C, while being constantly swirled and mixed. When the mixture was almost thawed, small lumps of ice being still visible, the vial was wiped, dried with tissue and transferred to the laminar flow hood. Thawing medium was gradually added to the cryovials whilst mixing gently. Thereafter, the cell suspension was transferred to a 15 ml Falcon tube containing thawing medium and the remaining medium added over the next five minutes while being mixed gently, allowing the DMSO to diffuse out of the cells. All the procedures were carried out under laminar flow hood.

The tube was centrifuged at 400 g for 10 minute, resuspended in washing medium, and centrifuged another 10 minutes at 1500 rpm. The supernatant was removed and the cell pellet resuspended in 1 ml of CCM-1 then counted.

2.6. NK precursor frequency (NKpf) cytotoxicity assay

2.6.1. Principle

The cytotoxic function of NK cells is usually determined by methods based on the release of different markers from the lysed target cells. In general, these markers are compounds containing radioactive isotopes such as 51 chromium, 75 selenium, or titanium. In the 1960s, Goodman introduced an in-vitro method to determine cytotoxic function of cells based on released radioactive 51 chromium (^{51}Cr) from lysed target cells. The ^{51}Cr release assay is one of the most commonly used methods that is easy to perform, highly sensitive, non-toxic to the cells and gives low spontaneous release (Holm, 1967; Brunner et al., 1968). The method does not change the morphology or the characteristics of the target cells and detection is easy and sensitive and was extended to cover other cytolytic reactions (Berke and Amos, 1973; Kemp and Berke, 1973; Stulting and Berke, 1973).

However, the use of ^{51}Cr has some well-known drawbacks, such as: short life of labelling reagent; a radioactive waste problem; slow release making incubation time long (usually 4 hours) because ^{51}Cr binds to cellular proteins in target cells in its ionic form, CrO_4^{2-} and ^{51}Cr is only released when target cells are sufficiently damaged; health hazards that increase risk to working personnel; cumbersome equipment; and emission of gamma rays.

This situation led to the development of a non-radioactive cytotoxicity assay using "Europium (Eu^{3+})" that was described for the first time by Blomberg and his co-workers in 1986. The detection of the released marker is based on time-resolved fluorometry, where the fluorescence from long-lived fluorescent probes is measured after short-lived background fluorescence has decayed. After release of the complex from lysed target cells, Eu^{3+} is chelated with a β-diketon known as enhancement solution. This chelate is highly fluorescent and can be quantified by time-resolved fluorometry. The chelate reduces the background fluorescence and enhances the sensitivity of the assay. Also, the

fluorescent decay time of Eu chelates is in the range of 100-1000ns compared to 10-20ns decay times for fluorescent biological samples (Blomberg et al., 1986b; Blomberg et al., 1986a; von_Zons et al., 1997).

The method is simple, sensitive, specific and rapid (2-3 hours incubation time) because Eu-DTPA is an inert chemical complex that does not bind to cellular proteins in target cells, and there are no health hazards. Finally, for the measurement of NK cell activity in many samples such as a population study, a cytotoxicity assay using non-radioactive reagents is desirable. This method has been used to measure human NK cell activity, cytotoxic T lymphocyte (CTLs) activity, lymphokine-activated killer (LAK) cell activity and complement-mediated cytolysis (Hemmila et al., 1984; Blomberg et al., 1996; Nagao et al., 1996; Haque et al., 2001).

2.6.2. Limiting dilution analysis (LDA)

The limiting dilution analysis (LDA) allows the frequency of a particular cell in a population of cells to be estimated per unit volume of solution. LDA is used in many areas of bio-research including immunology, bacteriology and cell-biology. LDA differentiates between a positive or negative response in each well of an experiment. The proportion of negative wells is used to estimate the cell frequency. A negative well assumes that there is no cell of the type being measured. By contrast, a positive well demonstrates that there is one or more such cells. The positive wells cannot be used to estimate the cell frequency because it is unclear how many cells give the response in each individual well (Strijbosch et al., 1987; Strijbosch et al., 1988).

The percent of the replicated wells at each responder cell dilution that were negative was recorded and plotted against the responder cell concentration. The point on the plot where 37% of the wells were negative corresponded to an average of one NK cell per well. On the basis of this assumption, the NKpf was calculated, using a computer program. This gave the mean NKpf plus 95% confidence intervals. It also calculated the goodness of fit (g.o.f). If g.o.f was >

15, results could not be attributed to single hit kinetics, i.e., probably more than one cell type was responsible for cytolysis (Strijbosch et al., 1987; Strijbosch et al., 1988).

2.6.3. Materials

a) Human AB serum (Sigma)

AB serum from healthy male individuals was heated at 56^0C for 30 minutes. This was aliquotted and stored at -20^0C.

b) Human AB serum (Quest)

AB serum pooled from non-transfused healthy male individuals had been heated and sterile filtered by Quest-Bio medical. This was aliquotted and stored at -20^0C.

c) Complete culture media (CCM-2)

Complete culture medium (CCM-2) consisted of RPMI 1640 (Sigma) supplemented with 10% heat inactivated human AB serum from normal healthy male donors (Sigma) and 3mM L-glutamine (Sigma). Antibiotics were not added to CCM-2 as this reduced assay sensitivity.

d) Recombinant Interleukin-2 (rIL-2)

Each vial of rIL-2 (Eurocetus, Amsterdam) contained 18 x 10^6 IU = 3x 10^6Cetus unit. 3 ml of PBS were added to the vial and mixed well until dissolved. This gave a concentration of $1x10^6$ Cetus Units per ml, that was diluted further to 1:10 with PBS ($1x10^5$ Cetus unit per ml). This stock solution was stored at -20^0C. Prior to use in the culture a vial of stock solution was thawed and diluted further 1:10 by adding 1ml of stock to 9 ml of filtered RPMI and then storing at -20^0C. At this concentration ($1x10^4$ Cetus Units per ml) 1µl contained 10 Cetus Units.

e) Recombinant Interleukin-15 (rIL-15)

Each vial of rIL-15 (R&D, UK) contained 5μg of the lyophilised rIL-15. 1.0 ml of sterile phosphate-buffered saline containing 1% human serum albumin was added to the vial and mixed well until dissolved. The concentration of this stock was 10ng/ml. This stock solution was aliquotted and stored at -20^{0}C. For use, in the culture, the contents of each stored vial (20 μl) were added to 50ml RPMI to give a concentration of 10ng/ml.

f) Preparation of irradiated allogeneic stimulator cell pool

PBMC were prepared by Ficoll-Isopaque gradient from four healthy randomly selected apheresis donors. PBMC were pooled and then irradiated (30 Gy) using a Cobalt source.

g) Phosphate buffer saline (PBS)

Stock solution of 0.05 molar (M) concentration of each buffer was made up with 3.45g sodium di-hydrogen phosphate ($NaH_2PO_4 H_2O$), and 3.55-g di-sodium hydrogen phosphate (Na_2HPO_4) dissolved in 500 ml H_2O. Thereafter, 95 ml of NaH_2PO_4 plus 405 ml Na_2HPO_4 were mixed and 4.5g sodium chloride added. The pH was checked and adjusted to 7.4 and stored at 4^{0}C until use.

h) HEPES Buffer

50 mM HEPES (5.958 g), 93 mM sodium chloride (2.178 g), 5 mM potassium chloride (0.187 g) and 2 mM magnesium chloride $6H_2O$ (0.204 g) were weighed out and dissolved in 500 ml of distilled water. The pH was adjusted to 7.4 and stored at 4^{0}C.

i) Calcium chloride

I) 100 mM solution: 0.147 mg was dissolved in 10 ml of sterile distilled water.

II) 1M solution: 1.47 g was dissolved in 10 ml of sterile distilled water and stored at -20^{0}C until required.

j) *Diethylene triamino pentacetate (DTPA)*

98.33 mg of 25 mM DTPA was dissolved in 10 ml of 0.01M sodium hydroxide by heating in a water bath at 60-65^{0}C for 1 hour and storing at -20^{0}C until required.

k) Europium chloride ($EuCl_3$)

36.9 mg $EuCl_3$ was dissolved in 0.01M HCL and stored at -20^{0}C.

l) Dextran sulphate

100 mg of dextran sulphate was dissolved in 10 ml of sterile distilled water at 37^{0}C. This was divided into aliquots of 0.5 ml and stored at -20^{0}C until required (not filtered).

m) Triton

Triton x -100 was made up to 2% triton by dissolving in PBS solution and storing at 4^0 C until required.

2.6.4. Method

2.6.4.1. Setting up the NKpf assay

The principles of LDA are illustrated as follows. PBMC was titrated in seven two-fold dilutions in CCM-2. The two-fold cell dilutions ranged from a concentration of 4X10^4 and to 0.0625X10^4 PBMC per 100µl/well in a 96-well, round-bottomed Falcon, culture plate (i.e. 4X10^4, 2X10^4, 1X10^4, 0.5X10^4, 0.25X10^4, 0.125X10^4, 0.0625X10^4) using 24 replicates per dilution. The baseline control consisted of 100µl of CCM-2 medium alone without PBMC. The assay

plates were incubated for 1 to 10 days at 37^0C in 5% CO_2 in a humidified atmosphere. All the procedures were carried out under laminar flow hood.

2.6.4.2. Addition of rIL-2 and rIL-15

After adding the dilution of PBMC to the wells, the rIL-2 (Eurocetus, Amsterdam) in CCM-2 was added to cultures to give a final concentration of 25 to 50 Cetus units/ml. This implies 2.5-5.0 Cetus units/well. The rIL-15 (R&D, UK) was similarly diluted to a final concentration of 10 or 20 ng/ml, depending on the experimental conditions. Both rIL-2 and rIL-15 were added on day 0 when setting up the assay and during feeding on day 5. All the procedures were carried out under laminar flow hood.

2.6.4.3. Adding the irradiated allogeneic stimulator cell pool

The pooled irradiated PBMC were added in a single concentration of 50,000 cells per 100 μl/well to responders on day zero and incubated at 37^0C in 5% CO_2 in a humidified atmosphere for 1,5 and 7 days.

2.6.4.4. Incubation time and feeding cultures

The duration of incubation varied through18 hrs, 5, 7 or 10 days. The 7 and 10 days assays were refreshed with CCM-2 on day 5.

2.6.4.5. Culture and maintenance of K562 cell lines

The HLA negative NK sensitive human erythro-leukaemic cell line, K562 (ECACC, UK) was used as a target for quantitating NKpf. The cell line was grown in stationary suspension cultures consisting of RPMI 1640 supplemented with 2% AB serum (Sigma) and 3mM L-glutamine (Sigma) in vented tissue culture flasks (25 cm^2, Falcon). To ensure optimal growth, culture media were replaced on the second day and harvested on days 4 to 5. The cell line was regularly screened for mycoplasma (Mycloplasma Test Medium Kit, Sigma). All the procedures were carried out under laminar flow hood.

2.6.4.6. Eu-labelling of K562 target cells

The K562 cell line was transferred from culture flasks into a 50 ml polypropylene tube (Falcon) and centrifuged at 400g for 10 minutes in the cold temperature (4^0C). Cells were washed twice with PBS. The approximate cell counts were performed after the first wash to calculate the amount of europium to be added. Labelling buffer consisted of 1mL HEPES buffer (Sigma), plus 40µl of a solution of 100µM Diethylene triamino pentacetate (DTPA, Sigma), plus 20µl of a 20µM solution of europium chloride ($EuCl_3$, Fluka). Then one ml of labelling buffer and 50µl of a solution of 500mM dextran sulphate (Fluka) were added to each $5x10^6$ K562 cells to increase cell permeability and allow the Eu-DTPA complex to enter the cell. The cell suspension was incubated for 15 minutes at 4^0C and gently, but thoroughly mixed between incubations. After 15 minutes, 30µl of a 100mM solution of $CaCl_2$ was added to stop the labelling reaction and then incubated for a further 10 minutes at 4^0C. After a total of 25 minutes incubation, the K562 cells were washed four times with a washing buffer containing, RPMI 1640, 3mM L-glutamine and 79µl/50ml of 1M $CaCl_2$ and centrifuged at 300g for 6 minutes at 4^0C. Thereafter, they were washed twice with CCM-2 and centrifuged at 400g for 6 minutes at 4^0C. Finally, the Eu-DTPA labelled cells (Eu-K562) were re-suspended in CCM-2 and adjusted to the desired count for cytotoxicity assays. All the procedures were carried out at 4^0C.

2.6.4.7. Quantitation of cytotoxicity

The last stage of the NKpf assay was performed by adding 5,000 to 10,000 Eu-K562 in 100µl to each well in a 96-well culture plate. At the same time as each cytotoxicity assay, a control plate was set up to control the reagents used and to determine spontaneous release. The control plate was set up with16 replicates for each reagent used in the assay including, CCM-2 only, triton only, enhancement solution only, CCM-2 plus triton, Eu-K562 plus CCM-2 and Eu-K562 plus triton.

All plates were briefly centrifuged at 200g at room temperature for one minute to accelerate the reaction, and then incubated for 3 hours at 37^0C in 5% CO_2 in humidified atmosphere. After incubation, plates were again centrifuged for 5 minutes at 600g at room temperature (RT).

Thereafter, 20μl aliquots of supernatant from each well of the assay plate were transferred to a new reader plate (Nunc) pre-filled with 200μl of enhancement solution. Finally, Eu-DTPA release was read in counts per second (c.p.s.) in a time resolved fluorometer (Delfia, Finland). The spontaneous release was determined by incubating target cells with CCM only, and maximum release was obtained by lysing the target cells with 2% Triton X-100. The percent spontaneous release was calculated by dividing spontaneous release by maximum release and multiplying by 100. If spontaneous release was > 15% assays where discarded.

The fluorometer reading was in c.p.s.. The mean and standard deviation (SD) between replicate wells of the baseline culture was calculated. Any test-well that exceeded the baseline plus three times the SD was scored *"positive"* and any test-well value below baseline plus three times the SD was scored *"negative"*.

In summery, using the principle of LDA, an NKpf assay was performed by the Eu release method. This fluorescence assay is used for quantitation of natural killer cell precursor. The K562 target cells were labelled with Eu-DTPA chelates, a complex that is chemically inert. The Eu-DTPA has the advantage of being relatively small and therefore able to leave the target cells just after they are rendered permeable by exposure to Perforin/Granzymes produced by responder cells.

2.7. Cytokine analysis by ELISA

2.7.1. Principle

A solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay) was used to measure the cytokine levels. The method was designed in the early 1970s and has been shown to be a sensitive technique for the rapid detection of serum proteins, circulating antibodies, and cell-surface antigens and cell products. The specific monoclonal antibody for each cytokine, called a capture antibody (Ab), is coated to a 96-well immunosorbent plate. The samples and standard dilutions were added to appropriate wells along the plates and any specific cytokine (Ag) present in the samples bound to the coated specific antibody. The second antibody acting against the one measuring specific cytokine was added to each well. If the first Ag-Ab complex developed, the second complex Ab-Ag-Ab (sandwich) was produced (Beech et al., 1997; Ishijima and Suzuki, 1998).

The supernatants of parallel cultures for pre and post-operative samples were collected after 5 days culture containing CCM-2, 25 Cetus unit/ml rIL-2 and 10 ng/ml rIL-15, and kept at -80^{0}C until required. The ELISA assay was used to measure IL-10, IL-4 and IFNγ.

2.7.2. Materials

a) Bicarbonate Buffer

0.636 g Na_2CO_3 (Sigma) and 1.172 g $NaHCO_3$ (Sigma) were dissolved in 400 ml distilled water, adjusted to pH 9.6 and stored at 4^{0}C until required.

b) Coat plate for IFNγ ELISA assay

15μg of IFNγ capture antibody solution (Pharmingen, CA) was added to 5ml bicarbonate buffer then 50 μg of this solution multi-pipetted across the Nunc immunobsorbent plates (Nalge Nunc, Denmark). The plates were gently tapped

to ensure antibody solution completely covered the base of the well. The plate was covered with a lid or sealing strip and stored at 4^0 C until required.

c) Coat plate for IL-10 ELISA assay

35μg of IL-10 capture antibody solution (Pharmingen, CA) was added to 5ml bicarbonate buffer then 50 μg of this solution multi-pipetted across the Nunc immunobsorbent plates. The plates were gently tapped to ensure the antibody solution completely covered the base of the well. The plate was covered with a lid or sealing strip and stored at 4^0 C until required.

d) PBS/0.1% tween solution

10 g PBS (Sigma) was weighed out and dissolved in 1000 ml of distilled water then 3ml tween (Sigma) added and mixed and stored at RT until required.

e) PBS/1% Bovine serum albumin (BSA) solution

1 g PBS (Sigma) and 1 g BSA (Sigma) were dissolved in 100 ml of distilled water and stored at RT until required.

f) Preparation of reference solutions for in IFNγ assay

15 minutes before use, cryopreserved stock tubes of IFNγ (Pharmingen, CA) were removed from -80^0 C thawed and diluted as described below:

For the top reference standard, 75μl of IFNγ stock was added to 175 μl RPMI (Sigma) at a final concentration of 30ng/ml. For the high quality control, 10μl of IFNγ stock was added to 490 μl RPMI to give a concentration of 2ng/ml. For the low quality control, 2.5 μl of IFNγ stock was added to 497.5 μl RPMI to give a concentration of 0.5ng/ml. All solutions were mixed gently before adding to the assay.

g) Preparation of reference solutions for IL-10 assay

15 minutes before use, the stock tubes of IL-10 (Pharmingen, CA) were removed from -80^0 C and thawed and diluted as described below:

For the top reference standard, 25µl of IL-10 stock was added to 225 µl RPMI (Sigma) to give a final concentration of 10ng/ml. For the high quality control, 5µl of IL-10 stock was added to 495 µl RPMI to give a concentration of 1ng/ml. For the low quality control, 1 µl of IL-10 stock was added to 499 µl RPMI to give a concentration of 0.2ng/ml. All solutions were mixed gently before adding to the assay.

h) Preparation of detection antibody solution for IFNγ assay (per plate)

15 minutes before use, 17.5 µl of the biotinylated anti-IFNγ antibody (Pharmingen, CA) was removed from 4^0 C, then added to 5 ml PBS/ 1% BSA solution and mixed well.

i) Preparation of detection antibody solution for IL-10 assay (per plate)

15 minutes before use, 15 µl of the biotinylated anti-IL-10 antibody (Pharmingen, CA) was removed from 4^0 C, then added to 5 ml PBS/ 1% BSA solution and mixed well.

j) Streptavidin peroxidase (per plate)

15 minutes before use, 5 µl of the Streptavidin peroxidase (Sigma) was removed from 4^0 C, then added to 5 ml PBS/ 1% BSA solution and mixed well.

k) TMB (3,3,5,5 tetramethyl benzidine) /DMSO

100 mg TMB (Sigma) was weighed out and dissolved in 10 ml of DMSO (Sigma) then aliquotted at 0.5 ml per bottle and stored at -20^0 C until required. The final concentration of this stock was 10mg/ml TMB in DMSO.

l) Phosphate Citrate Buffer

3.6459 g Na_2HPO_4 (Sigma) and 2.553 g Citric acid.H_2O (Sigma) were weighed out and dissolved in 500 ml distilled water and stored at 4^0C until required.

m) TMB/DMSO/H_2O_2/phosphate citrate solution (per plate)

3 μl H_2O_2 (Sigma) and 100 μl of thawed TMB/DMSO were added to 10ml phosphate citrate (Sigma) mixed well and kept at RT until required.

2.7.3. Method (measurement of IFNγ and IL-10)

Nunc immunosorbent plates (Nunc, Denmark) coated with captured antibodies specific for either IFNγ or IL-10 (Pharmingne, CA) was stored at 4^0C overnight. Plates were then blocked with 200μl PBS 1% bovine serum albumin (Sigma) and kept at 37^0C for one hour. After the desired incubation time, plates were washed twice with PBS/0.1% tween, and twice with PBS. The left-over PBS in the plates prevented them from drying out before samples, standards and blanks were added. Standard dilution curves, and high and low quality controls, were prepared. PBS was emptied from the plates by decanting and tapping them on soft tissue. 50μl of diluent (RPMI) was applied to the standard wells in duplicate across the plates except for the top standard wells to make up the volume to 100μl. 100μl of top standards for either IFNγ or IL-10 were transferred into the top standard wells. Rinsing the pipette tip allowed mixing of the well contents. 50μl of the top standard were aspirated, and added to the next wells. This serial dilution continued to the lowest concentration of the standard wells across the plates. Then, 50μl aliquots of cultured supernatant for test samples, blanks (RPMI), and high and low controls were transferred into appropriate wells along the plates and incubated for 2 hours at room temperature (RT). After incubation, the plates were rinsed four times by PBS/0.1% tween, and after the last time, they were decanted by tapping on soft tissue to remove any residual PBS/0.1% tween. They were then reacted with 50μl aliquots of detection

antibodies (Pharmingne, CA). Plates were left for one hour at RT then rinsed six times with PBS/0.1% tween, and after the last time decanted by tapping on soft tissue to remove any residual PBS/0.1% tween. 50μl streptavidin peroxidase (Sigma) was added to each well and left for a further 30 minutes at RT. Plates were rinsed six times and 100μl TMB/H_2O_2/phosphate citrate (Sigma) added. When the blue colour started to show in the penulimate standard, 50μl of 2M H_2SO_4 was added to stop the reaction. Finally, absorbance at 450nm within 30 minutes of stopping the reaction was read on the scanner (Dynatech, UK).

2.8. Measurement of IL-4

100μl of standard diluent was pipetted into each standard well across the coated plates in duplicate, except for the highest standard wells (30pg/ml), for which 100μl of the highest standard was added into the second top standard wells. Rinsing the pipette tip allowed mixing of the well contents. 100μl of standard was aspirated and serially diluted and continued to the lowest concentration of the standard wells to give a range between 30-0.47 pg/ml. Then, 100μl aliquots of culture supernatants, blanks and positive controls were transferred into appropriate wells along the plates in duplicate. Plates were covered with plate sealer and incubated for two hours at RT. The contents of the wells were decanted and the plates rinsed four times with PBS/0.1% tween, and after the last time, decanted by tapping on soft tissue to remove any residual PBS/0.1% tween. 100μl of prepared working detector (Pharmingne, CA) was added to each well and plates were covered and left for another hour at RT. After incubation well contents were decanted and plates rinsed seven times with PBS/0.1% tween, and after the last time, they were decanted by tapping on soft tissue to remove any residual PBS/0.1% tween. 100μl of prepared substrate solution was pipetted into each well and the plates incubated at RT for 30 minutes. After incubation 50μl of stop solution was added to each well. Finally, the absorbance at 450nm was read on the scanner (Dynatech, UK) within 30 minutes of stopping the reaction.

2.9. Immunophenotyping studies

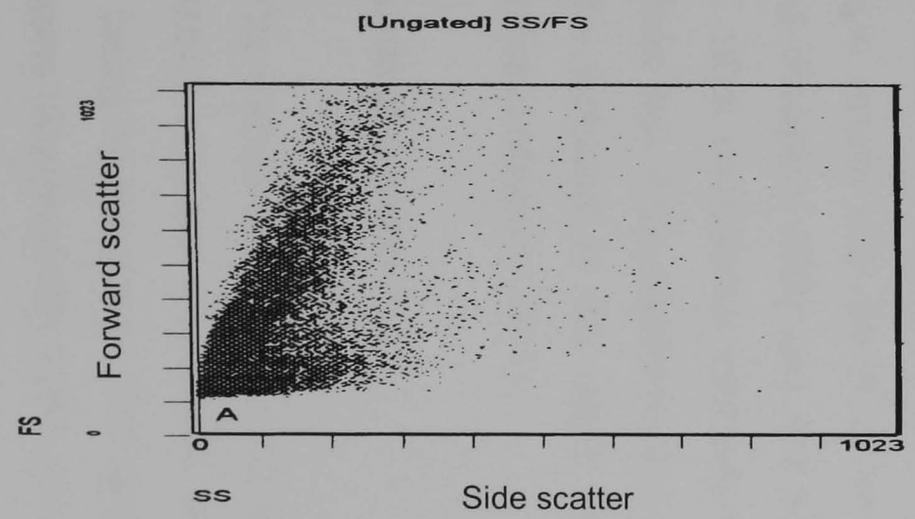
The PBMC were set up in parallel cultures under various conditions mentioned above. At 5 days, cells were collected from culture plates and adjusted to $1x10^6$ cells per ml in RPMI 1640. 5μl (1μg) of each antibody was added to 100 μl of cell suspension at $1x10^5$ / ml, and mixed. The cell/antibody mixture was incubated at 4^0C for 30 minutes, washed once at 700g for 10 minutes and re-suspended in 300μl of RPMI 1640. Finally, the flow cytometric analysis was performed on a Coulter Epics®XL (Luton, UK).

Monoclonal antibodies specific for the human cell differentiation (CD) marker were used for two-colour flow cytometric analysis of CD3-CD56+, and CD3-CD16+ cells. Monoclonal antibodies directly conjugated to fluorochromes and controls were CD56 fluorescein isothiocyanate (FITC) (Becton Dickinson, UK), CD16 FITC (DAKO), CD3- phycoerythrin (PE) (DAKO) and FITC neg. (DAKO). The CD16+ and CD56+ population was calculated as a percentage of the total CD3- cell population by flow cytometric analysis. Figure-7.2 demonstrates the gates that were used for enumeration of CD3-CD56+ cells by flow cytometry. Cells of interest were defined as those expressing CD56 or CD16 in the CD3 negative population. These were selected using electronic gating with double staining by combined antibodies against the CD3 and CD56 or CD3 and CD16. The CD3- cells were selected by drawing the region (B) horizontally against the region (A) in gate-2 and only the cells in region (B) were analysed for the next step. After identification of CD56+ or CD16+ cells in the CD3- population, identified cells were selected in gates-3 by drawing region (C) vertically against the gate-3 in CD3- population. A histogram of CD56 or CD16 expression was shown in region (D) in gate-4. Cells in region (C) or (D) were analysed as percent CD56 or CD16 positive cells in CD3- population.

Stem Cell Laboratory
Department of Transplantation Sciences
University of Bristol

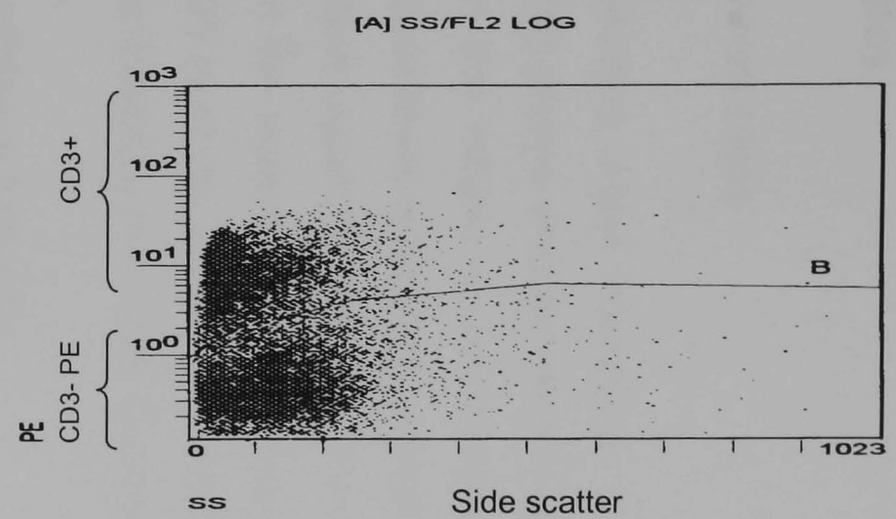
[Ungated] Legend
BECKMAN COULTER EPICS XL
06:JUN:01
11:59:15

Protocol Ahmad CD56FITC



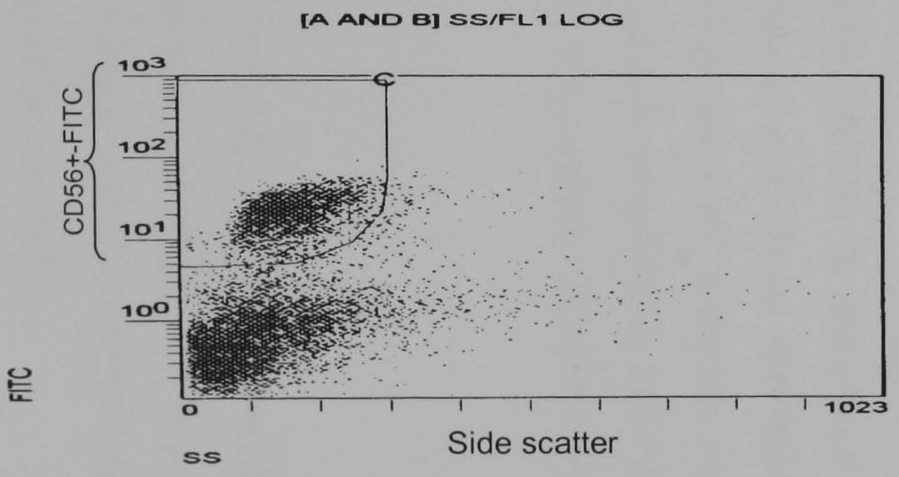
[Ungated] SS/FS

Region	Number	%Gated
A	22127	93.80



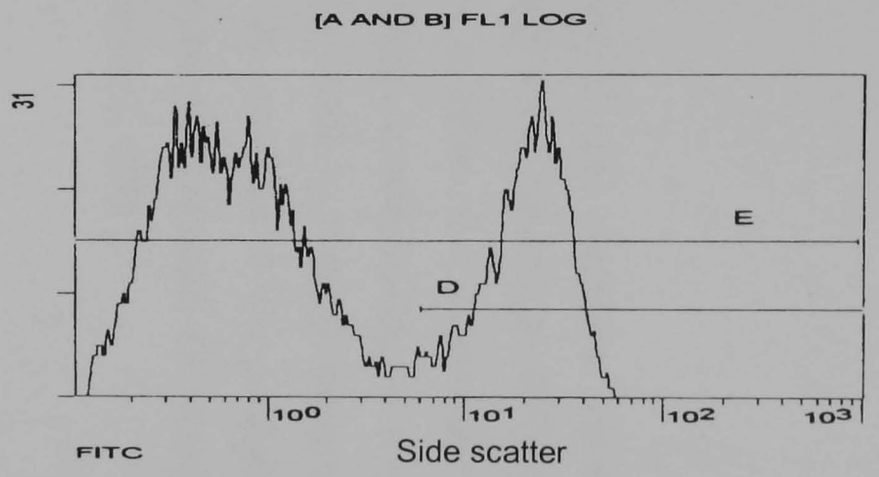
[A] SS/FL2 LOG

Region	Number	%Gated
B	10545	47.66



[A AND B] SS/FL1 LOG

Region	Number	%Gated
C	3564	33.80



[A AND B] FL1 LOG

Region	Number	%Gated
D	3677	34.87
E	10545	100.00

Figure 7-2: Schematic diagram of different gates using for enumeration of CD3-CD56+ cells. Flow cytometry dot plots gated on CD3- cells (B) show populations defined by reactivity with CD56 MAb (C). Upper left quadrant forward versus side scatter; upper right, CD3- cells (inside gate B); lower plots show expression of CD56 positive cells in CD3- population gated vertically by B. Lower left shows a dot-plot of CD56 against side scatter; lower right is a histogram of CD56 expression in CD3- population.

Section 3.

Statistics

3.1. Student *t*-test

Student *t*-test compares the means of two samples between two underlying groups whether there is a significant or not significant of the difference between the means of two samples. The p-value (the probability value or significance level) is used to determine significant or non-significant of results. If the p-value equals or less than 0.05 ($p<0.05$), there is a significance difference between the means of two samples group. By contrast, if the p-value is more than 0.05 ($p>0.05$), there is no significant difference between the means of two samples (Bradford Hill, 1988; Campbell and Machin, 2001).

3.2. Confidence intervals

Confidence intervals (CI) indicates the precision of study estimates as population values and the width of confidence intervals is associated with the standard deviation (SD) and the sample size of a normally distributed set of values. 95% confidence intervals (95% CI) is the most common choice that determines that, if the experiment is repeated one hundred times 95 of the values would be included in the upper and lower parameters (Bradford Hill, 1988; Campbell and Machin, 2001).

3.3. Standard deviation

The standard deviation (SD) is a measure of the variability or scatter of the observations between individuals, around the average value (the mean) of the sample being investigated. The standard deviation is usually signed as '$\pm SD$' in publications (Bradford Hill, 1988; Campbell and Machin, 2001).

3.4. The correlation coefficient

The correlation coefficient (r-squared value) measures the relation between two or more variables in a group of subjects. The correlation coefficient is a number between –1 and +1. The correlation coefficient is signed as "r" in publications. The value between zero and one ($0<r<+1$) indicates there is a positive linear relationship between two characteristics of a population (r is positive). Thus two characteristics are positively correlated implying that low or high value of one characteristic of a population correlate with low or high level of another characteristic in same population respectively. By contrast, a value between zero and minus one ($-1\geq r<0$) indicates there is a negative correlation (r is negative) between two characteristics of a population. Thus negative 'r' implies that low value of one characteristic of a population correlates with high level of another characteristic in the same population and vice-versa. Finally, a value equal to zero ($r=0$) indicates there in no correlation between two values implying that low or high value of one characteristic of a population do not correlate with low or high level of another characteristic in same population being investigated within a population (Bradford Hill, 1988; Campbell and Machin, 2001).

3.5. Analysis of limiting dilution data

The mean and standard deviation Eu-release of the control containing only CCM-2 medium and Eu-K562 without PBMC are calculated. If the value of each Eu-release assay wells is less than the mean plus three standard deviation (mean+3SD) of Eu-release of the control wells, these assay wells are scored as negative. Then the numbers of negative wells within each replicate and the total number of replicates are used to determine the frequency of a particular cell (NKp) in a population of cells (PBMC). The numbers of negative assay wells are recorded and plotted against the responder cell concentration and the numbers of replicates. The point on the plot where 37% of the wells are negative corresponds to an average of one particular cell (e.g. NKp) per well. Using this assumption, the NKpf was calculated in this study with a computer program

based on the generalised linear interactive modelling (GLIM) software package (Taswell, 1981; Strijbosch et al., 1987).

3.6. Median

The median is a particular value that equally divides the distribution of a variable. Thus median determines that the half of the values of a variable are greater than the median, and half of the values are less (Bradford Hill, 1988; Campbell and Machin, 2001).

Section 4.

Results

4.1. Development a method for quantifying natural killer precursors (NKp)

The frequency of natural killer cell precursors (NKpf) was measured in human peripheral blood mononuclear cell (PBMC) samples, where functional maturity was reflected in lysis of K562 target cells. Initial experiments defined the factors influencing the NKpf. A method for quantitating the absolute number of natural killer precursor (NKp) cells was then developed. The technique was based on the principle of limiting dilution analysis (LDA). The NKpf assay was set up with a range of cell dilutions from 40,000 to 625 per 100μl/well in 96-well culture plates in different culture conditions. At the end of the culture period, the K562 cell line labelled with Europium (Eu-K562) was added and Eu release was measured in culture supernatants using time-resolved fluorometry. To avoid individual variation during the development of the technique, venous blood was taken from one healthy adult (AG) at different times. The NKpf estimates were expressed as a mean of 24 replicates with 95% confidence intervals (95%CI). The significance of the difference between NKpf results for each group was analysed with the two-tailed student 't' test. Results were expressed as a mean NKpf with 95% CI estimate plotted on a Log_{10} scale with 95% confidence intervals.

4.1.1. Effect of culture duration and timing of rIL-2 addition

These experiments were performed to define the influence of added rIL-2 and culture duration on NKpf. Figure-8.4 shows the results obtained from 3 to 7 different experiments. PBMC were cultured in CCM-2 containing heat inactivated Sigma-AB for 1, 5, 7 and 10 days incubation in the absence and presence of rIL-2 at 25 Cetus units/ml. The rIL-2 was added on the day of set-up (day 0) of the assay and during feeding on day five.

Culture in the absence of IL-2, resulted in the rapid decrease of the NKpf (per 10^6 PBMC) from 63± 6 on day one, 10±4 on day five, 3±1 on day seven and 4±2 on day ten. NKpf was almost immeasurable after seven days (fig-8.4a). By contrast, when rIL-2 was added, NKpf increased rapidly from 105±4 on day one, 800±145 on day five, 1026±206 on day seven and 1255±123 on day ten. The increment between days one and five implied rapid maturation of non-functional precursor (NKp). The small but significant increment in NKp numbers between days five and ten after adding extra rIL-2 on day five implied that even at this time cultures contained NKp at different stages of maturation ($p<0.004$ between days 5&10) (fig-8.4b).

The lower limit of detection was one NKp per 10^6 PBMC (fig-8.4a). The maximum NKpf detected after seven days of culture was 2,450 NKp per 10^6 PBMC (0.25%), obtained from experiments with rIL-15 (see fig-12.4 below).

4.1.2. Effect of extra rIL-2 on day 5

These experiments were designed to observe the effect of extra rIL-2 added during feeding on day five. Figure-8.4c shows the results obtained from 4 different experiments, when PBMCs were cultured for 5, 7 and 10 days after adding 25 Cetus units/ml rIL-2, only on day 0. There was no significant difference between NKpf estimates between days five and ten (776±151 on the five, 816±174 on day seven and 821±198 on day ten). By contrast, when extra rIL-2 (25 Cetus unit/ml) was added, on day five, the NKpf rose from 761±160 at five days to 1043±137 at seven and 1134±131 at 10 days (fig-8.4d). There was no significant difference between days 7 and 10 but both differed from the day 5 result ($p<0.005$) (fig-8.4d).

However, the absence of a significant difference between days five and ten, without additional rIL-2 on day 5 suggested that a fraction of late maturing NKp became IL-2 sensitive during the latter period of culture (fig-8.4c). This was confirmed when additional rIL-2 was added on day five in a second experiment in which NKpf was significantly augmented between five and seven days ($p<0.004$)

(fig-8.4d). In most cases, NKpf reached a plateau between seven and ten days ($p>0.4$), suggesting that all available rIL-2 sensitive precursors had been activated (fig-8.4c-d).

These results confirmed that the differentiation of NKp and maintenance in culture was dependent on the continued presence of rIL-2, and that the full potential NK function was reflected only in NKpf assays cultured to maturity over seven days.

Notwithstanding this observation, all ensuing assays were terminated at day five; the rationale being that of technical simplicity and greater sensitivity during the clonal expansion phase.

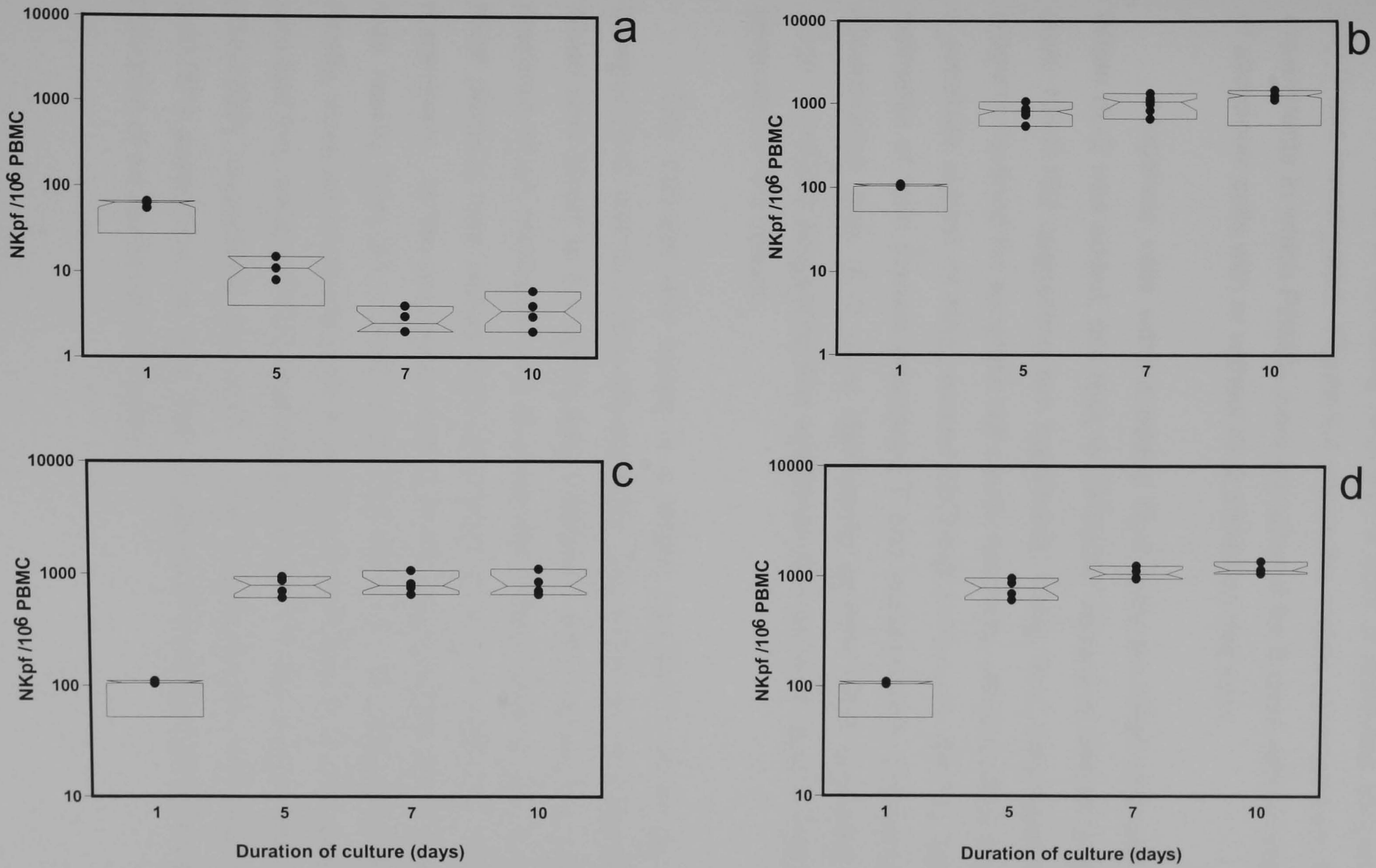


Figure-8.4: Mean NKpf (95% CI) plotted at different times of culture on a $\log_{10}$ scale ranging from 1-10^4 in a and b and 10-10^4 in c and d. ***a)*** no added cytokines, $p<0.05$ between day 1&5; ***b)*** rIL-2 added on days one and five together with fresh CCM-2, $p<0.05$ between days 1&5, 5&7, 5&10 and $p>0.05$ between day 7&10; ***c)*** rIL-2 added on day zero but only fresh CCM-2 on day five, $p<0.05$ between days 1&5, $p>0.05$ between days 5&7, 5&10 and 7&10; ***d***) rIL-2 added on day zero and day 5 in fresh CCM-2, $p<0.05$ between days 1&5, 5&7 and 5&10, $p>0.05$ between day 7&10. Results are from three to seven experiments with cells from AG.

4.1.3. Effect of allo-activation

The effect of activating NKp with a pool of irradiated allogeneic cells on day 0 was investigated. Figure-9.4 shows the results obtained from five different experiments in which PBMCs were co-cultured for 5 days with an irradiated pool of allogeneic cells with or without rIL-2 added on day zero.

Allogeneic cells without added rIL-2 gave an NKpf estimate of 190±82. When rIL-2 was added, this rose to 1919±307 compared with rIL-2 alone, which gave 1044±169 suggesting two hypothesis: firstly, rIL-2 may have rescued NK progeny destined for apoptotic cell death; secondly, allo-activation may stimulate a separate subset of NKp, termed NKT-cell precursors (NKTp) that have the hallmarks of both thymus processed T and non-thymically processed NK cells. Alloactivation plus rIL-2 gave significantly greater NKpf estimates than rIL-2 alone, ($p<0.05$) suggesting that additional differentiation signals may have been generated in the culture.

This concept was tested in a single experiment where the increment between rIL-2 and rIL-2 plus allo-activation was estimated on days one, five and seven and found to be significantly different. After one day of culture in the presence of an irradiated pool of allogeneic cells added on day zero only, the NKpf (95%CI) rose rapidly from 26 (18-34) to 83 (58-108) and 152 (113-192) respectively. In the presence of rIL-2 at 25 Cetus unit/ml alone NKpf (95%CI) rose rapidly from 55 (41-69) to 517(471-864) to 762 (629-899) respectively. Finally, when an irradiated pool of allogeneic cells and rIL-2 were added on day zero and five, NKpf (95%CI) rose rapidly from 86 (71-99) to 683 (593-775) to 877 (710-1025) respectively (fig-10.4). This increment persisted until day seven ($p<0.001$) supporting the view that an allogeneic stimulus was associated with activation of an additional cell subset.

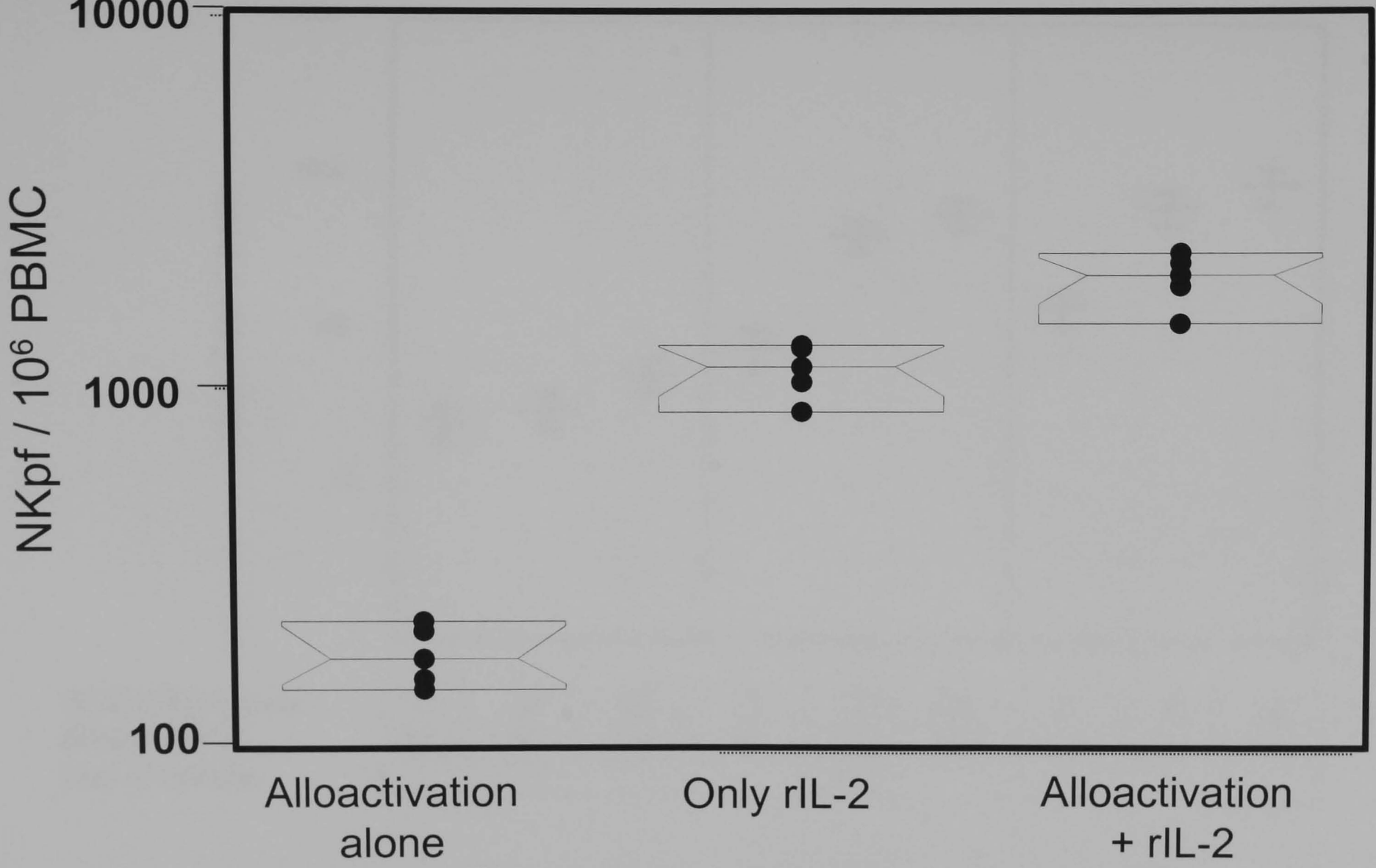
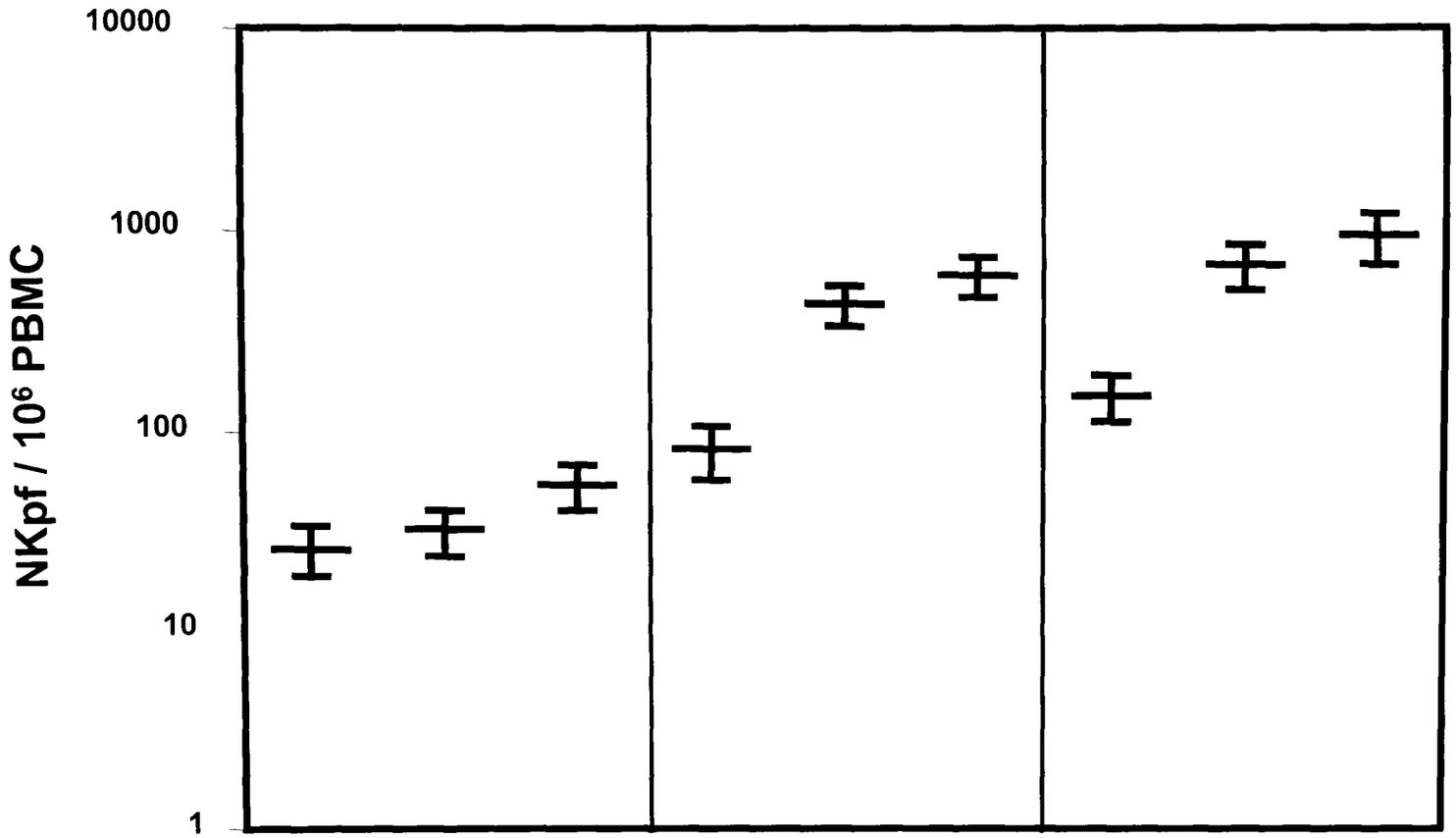


Figure-9.4: Mean NKpf (95% CI) after five days of culture plotted on a $\log_{10}$ scale ranging from 10^2-10^4 after co-culturing with an irradiated pool of allogeneic cells with or without added rIL-2 (25 Cetus units/ml) alone on day zero. $p<0.05$ between alloactivation alone and only rIL-2, between only rIL-2 and alloactivation+rIL-2. Results are from five experiments with cells from AG.



rIL-2 (Cetus units)	0	25	25	0	25	25	0	25	25
Alloactivation	Yes	No	Yes	Yes	No	Yes	Yes	No	Yes
Day of culture	1			5			7		

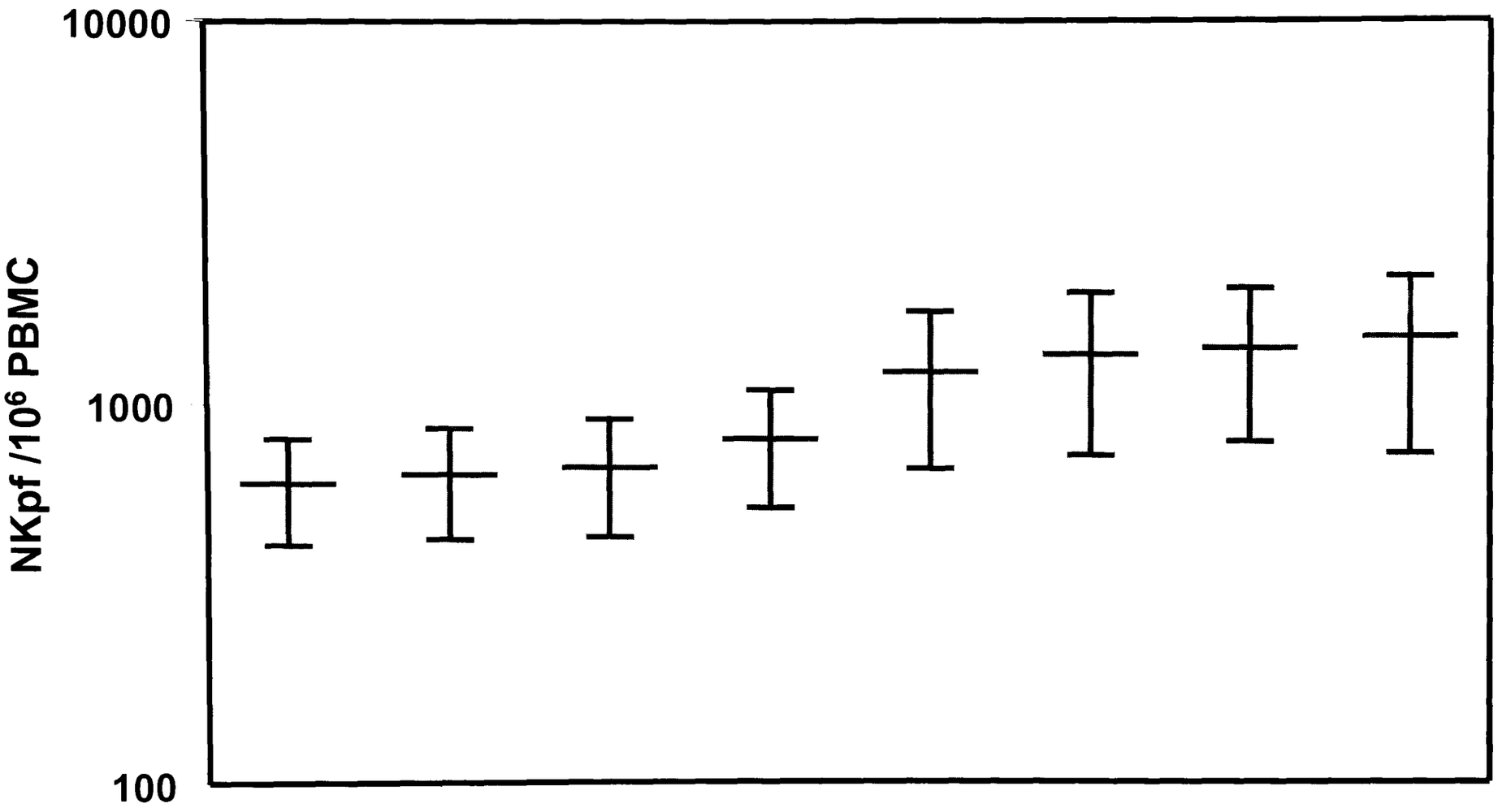
Figure-10.4: Mean NKpf (95% CI) after one, five and seven days of culture plotted on a $\log_{10}$ scale ranging from $1\text{-}10^4$ after co-culturing with an irradiated pool of allogeneic with or without added rIL-2 (25 Cetus unit/ml) on day zero and day 5. $p<0.05$ between days 1&5, 1&7 and 5&7 in all above conditions. Results are from one experiment with cells from AG.

4.1.4. Effect of rIL-15 on NKpf estimates

rIL-15 was a candidate cytokine that may have accounted for some of the allo-activation effect. Therefore, adding rIL-15 to the culture was investigated in the knowledge that it played a pivotal role in differentiation of NK cells from their progenitors and shared many functions ascribed to IL-2. Figure-11.4 shows the NKpf estimates with different concentrations and combinations of rIL-2 and rIL-15.

Thus NKpf estimates were highest, when PBMC were cultured with rIL-15 at 10-20 ng/ml. Indeed, these levels were significantly higher than with rIL-2 at 50 Cetus unit/ml alone ($p<0.05$). Furthermore, no incremental effect on NKpf resulted from the addition of rIL-2 to rIL-15.

Figure-12.4 shows the results obtained from 4 different experiments, for which rIL-15 alone, or rIL-15 plus rIL-2 gave significantly greater NKpf estimates than rIL-2 alone ($p<0.05$). Additionally, in presence of rIL-2 at 25 Cetus unit/ml NKpf ($\pm$SD) was 622$\pm$79, in presence rIL-15 at 10ng/ml 1924$\pm$180 and finally, in presence of rIL-2 at 25 Cetus unit/ml plus rIL-15 at 10ng/ml2014$\pm$229. This confirmed that rIL-15 alone is significantly ($p<0.05$) more effective in driving differentiation of NKp to maturity than rIL-2 alone.



rIL-2 (Cetus unit)	50	25	25	50	50	0	25	0
rIL-15 (ng/ml)	20	20	0	0	10	10	10	20

Figure-11.4: Mean NKpf (95% CI) after 7 days of culture plotted on a $\log_{10}$ scale ranging from 10^2-10^4 after adding different concentrations and combinations of rIL-2 and rIL15 on both day zero and 5. $p<0.05$ between rIL-2 at 25-50 Cetus unit/ml and rIL-15 at 10-20 ng/ml. Results are from one experiment with cells from AG.

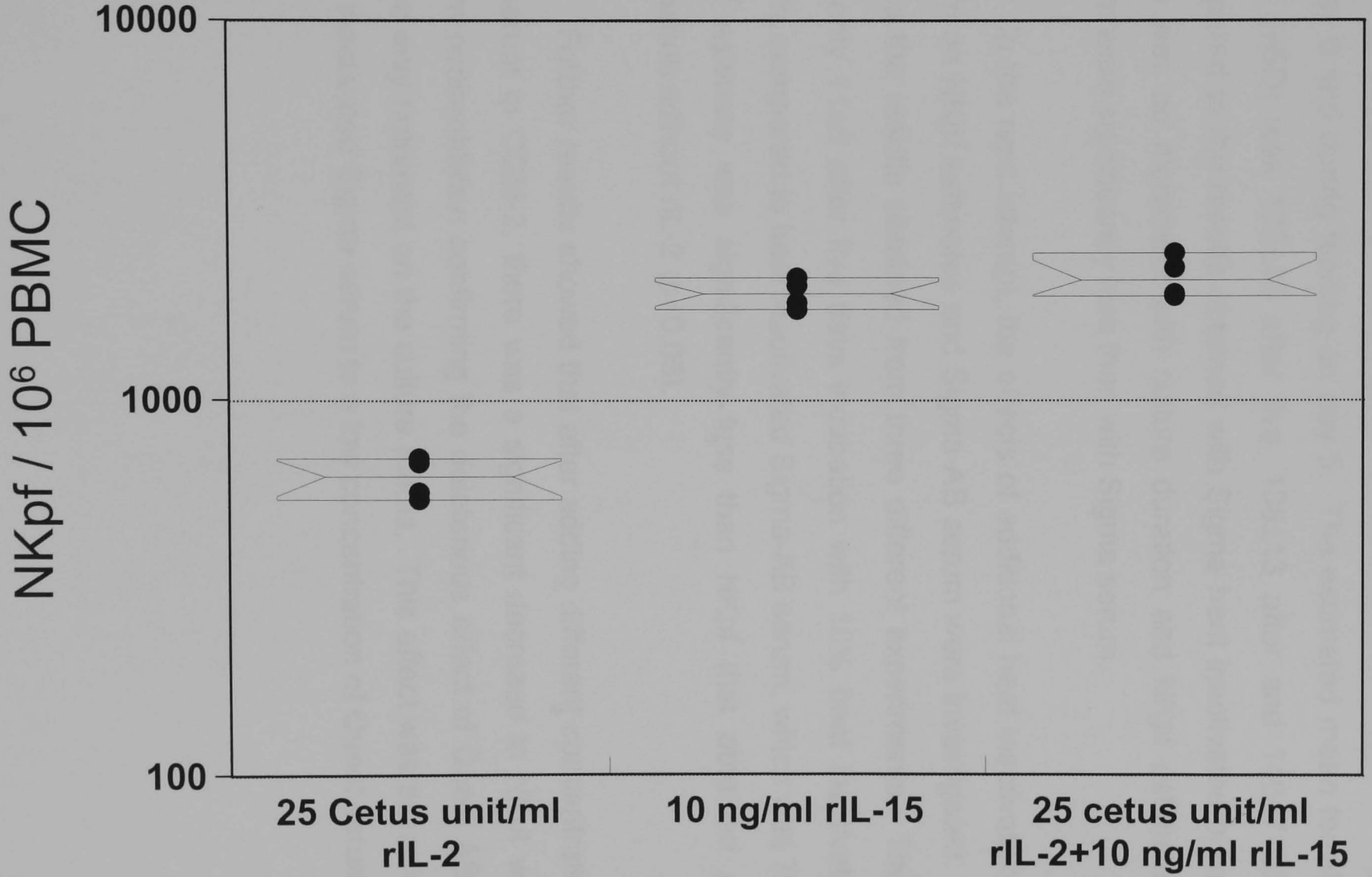


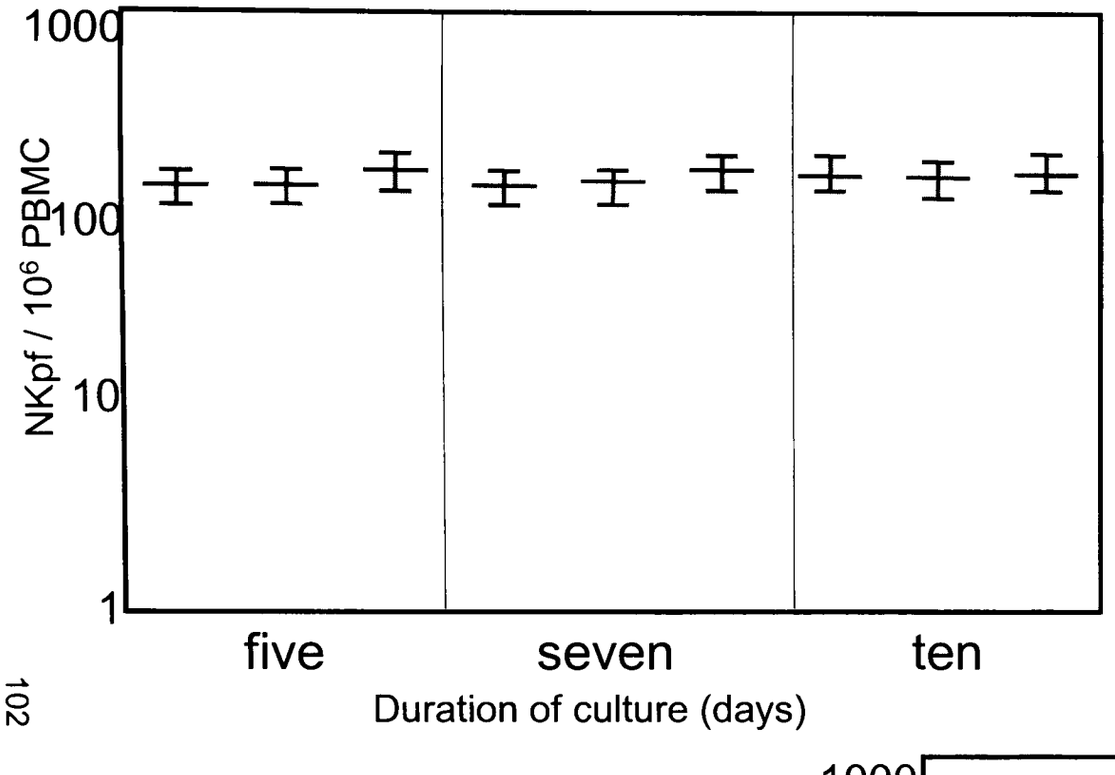
Figure-12.4: Mean NKpf (95% CI) after 5 day of culture plotted on a $\log_{10}$ scale ranging from 10^2-10^4 after adding different concentrations and combinations of rIL-2 and rIL15 on day zero as indicated. $p<0.05$ between only rIL-2 & only rIL-15, only rIL-2 & rIL-2+rIL-15 , and $p>0.05$ between only rIL-15 & rIL-2+rIL-15. Results are from four experiments with cells from AG.

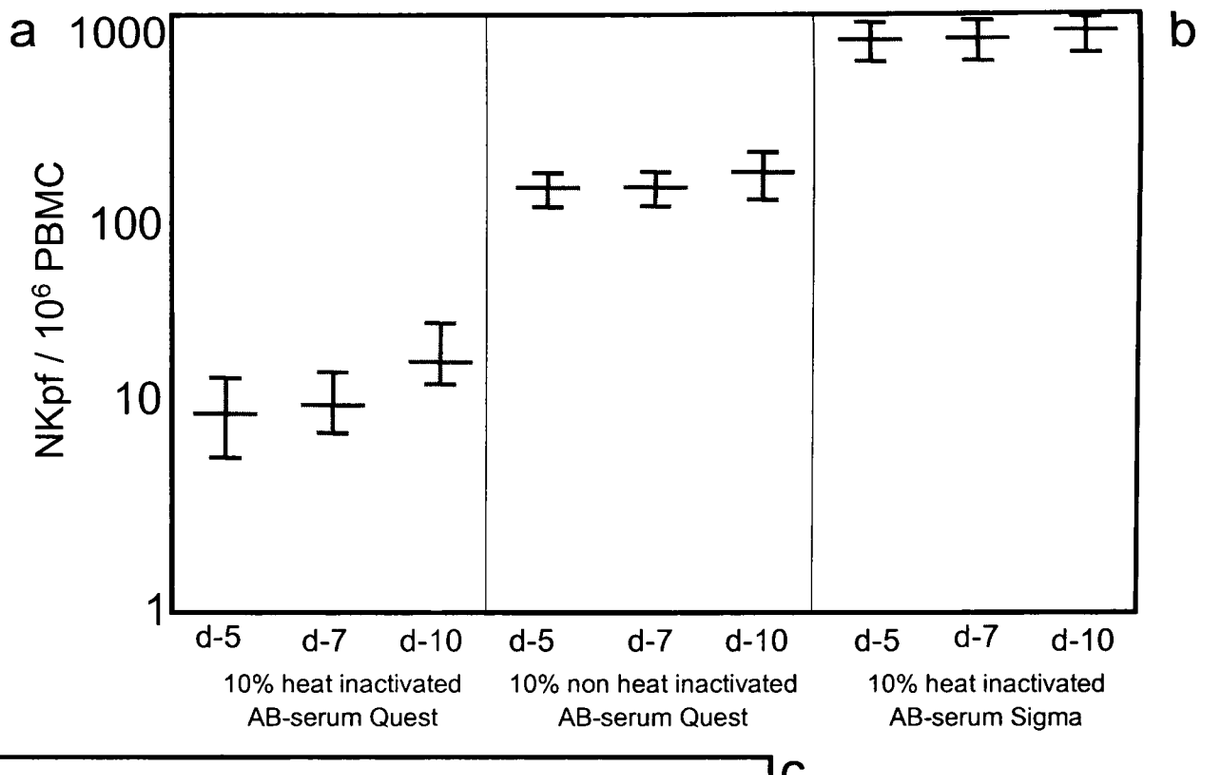
4.2.1. Effect of different source of human AB serum (Quest)

Figure-13.4a shows the results obtained from three different experiments where PBMC were cultured in CCM-2 containing heat inactivated human AB serum where the heat inactivation had been done by the company (Quest) for 5, 7 and 10 days in the presence of 25 Cetus units/ml rIL-2. The rIL-2 was added on day 0 and during feeding on day 5. The estimated mean for all experiments NKpf (±SD) was 132±15 after five, 138±13 after and 146±7 after ten days. Compared to the results obtained with Sigma heat inactivated human AB serum there was no increment with culture duration and NKpf estimates with Quest serum were significantly less than with Sigma serum.

In the next attempt, the effects of additional heat inactivation of Quest-AB serum on NKpf estimates and Sigma-AB serum were investigated. Figure-13.4b shows the results obtained from three different experiments. The NKpf (±SD) was only 11±6 after five days incubation with 10% heat inactivated Quest AB serum, compared to heat inactivated Sigma-AB serum, which was 781±160. This NKpf estimate was significantly less than NKpf that obtained after one-day incubation without rIL-2 ($p<0.05$).

Further results showed that after adding different concentrations of Quest-AB serum in CCM-2, there was a significant decrease in NKpf with increasing serum concentration confirming the deleterious effect of Quest AB serum, used as the only nutriment on the culture media. This effect was prevented by adding heat inactivated Sigma serum to a low concentration of Quest serum (fig-13.4c).





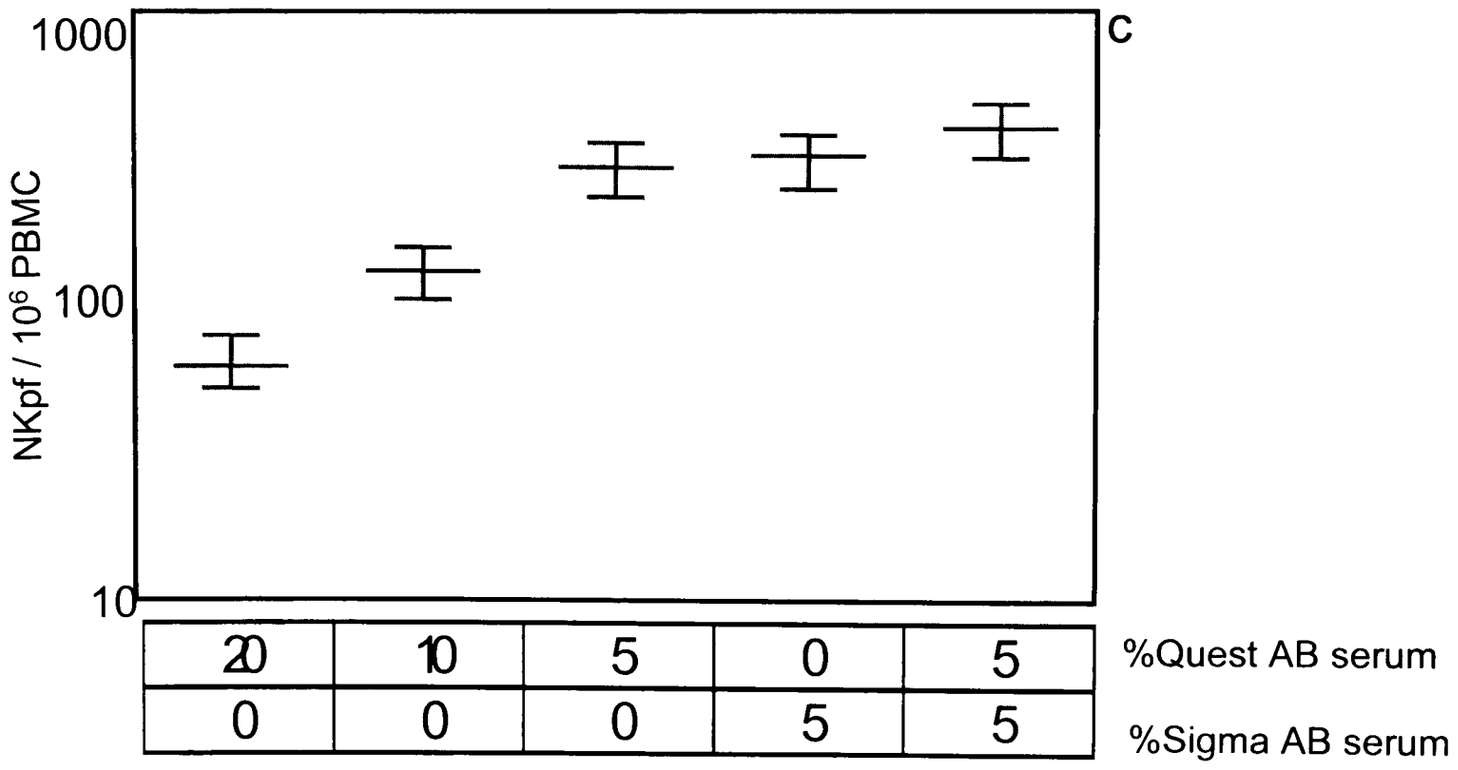


Figure-13.4: a) NKp (95% CI) plotted at different times of culture on a log 10 scale ranging from 1-10^3. PBMC were culture with 10% Quest-AB serum and 25 Cetus unit/ml rIL-2. **b)** NKp (95% CI) after 5 days of culture plotted on a log 10 scale ranging from 1-10^3. PBMC were cultured with 10% heat inactivated Quest-AB serum, 10% Quest-AB serum and 10% heat inactivated Sigma-AB serum in the presence 25 Cetus unit/ml rIL-2. **c)** NKp (95% CI) after 5 days of culture plotted on a log scale ranging from 10^1-10^3 after adding different concentrations and combinations of Quest-AB serum and heat inactivated Sigma-AB serum in the presence 25 Cetus unit/ml rIL-2. In all experiments cells were from AG.

4.2.2. Effect of the human bladder carcinoma cell line 5637 derived cytokines

The human bladder carcinoma cell line 5637 is a well-known source of haematopoietic cytokines and has been used to accelerate the growth of normal cells such as granulocyte and monocyte progenitors and malignant haemopoietic cells and cell lines. 5637 is an inexpensive source of cytokines used to maintain leukaemia cell lines in long-term culture. 5637 secretes granulocyte-stimulating colony factor (G-SCF), GM-CSF, macrophage-colony stimulating factor (M-CSF), SCF, IL-1β and IL-3 (Quentmeier et al., 1997; Ferrero et al., 1999).

To observe the effects of 5637 on NKpf estimates, PBMC were cultured with 5637 supernatant at 10 and 20% for five days without adding rIL-2 or rIL-15. The NKpf (±SD) estimates were 14±2 with 10% and 6±2 with 20% 5637 supernatant confirming that it is unable to act on NKp cells.

4.2.3. Effect of incubation time with Eu-K562

Adding Eu-K562 to each culture plate constitutes the final stage of the NKpf assay. The plate is centrifuged to accelerate the reaction and incubated at 37^0C in 5% CO_2 in a humidified atmosphere. In these experiments, plates were incubated for two or three hours at 37^0C in 5% CO_2 in a humidified atmosphere. Table-2.4 shows the results obtained from three different experiments, when plates were incubated for two and three hours.

Table-2.4: NKpf obtained from three experiments

Experiments	2h incubation	3h incubation
1	1117	950
2	1056	1242
3	800	781

$p>0.05$

Spontaneous release is the normal release of europium during the assay without added responder cells and the maximum release is that obtained by lysing the target cells with 2% Triton X-100. In the next experiment, the effect of incubation time on spontaneous release was measured (table-3.4). The percentage of spontaneous release was calculated by dividing spontaneous release by maximum release and multiplying by 100.

Table 3.4: The percent spontaneous release from eight experiments after 2 and 3 hours incubation of control plates

	Percent spontaneous release	
Experiments	2 hours	3 hours
1	9	14
2	10	16
3	11	12.5
4	13	16
5	13.5	11
6	15	12
7	15	13
8	15	14

$p>0.05$

The results obtained from both tables' shows that the plates in the final stage of the NKpf cytotoxicity assay could be incubated for two hours confirming that the Eu-label is released after shorter incubation time than radioactive ^{51}Cr assay (usually 4 hours).

4.2.4. Effect of number of Eu-K562 added

In the next experiments sensitivity of the assay to target cell number was investigated. 5,000 and 10,000 Eu-K562 were added in 100μl to each individual well of a 96-well culture plate. Table-4.4 shows the NKpf estimates obtained from four different experiments.

Table-4.4: NKpf obtained after adding 5000 and 10000 of Eu-K562.

Experiments	5,000 target cells/well (E:T ratio 8:1)	10,000 target cells /well (E:T ratio 4:1)
1	650	608
2	816	776
3	664	745
4	757	795

$p>0.05$

4.2.5. Effect of freezing on NKpf

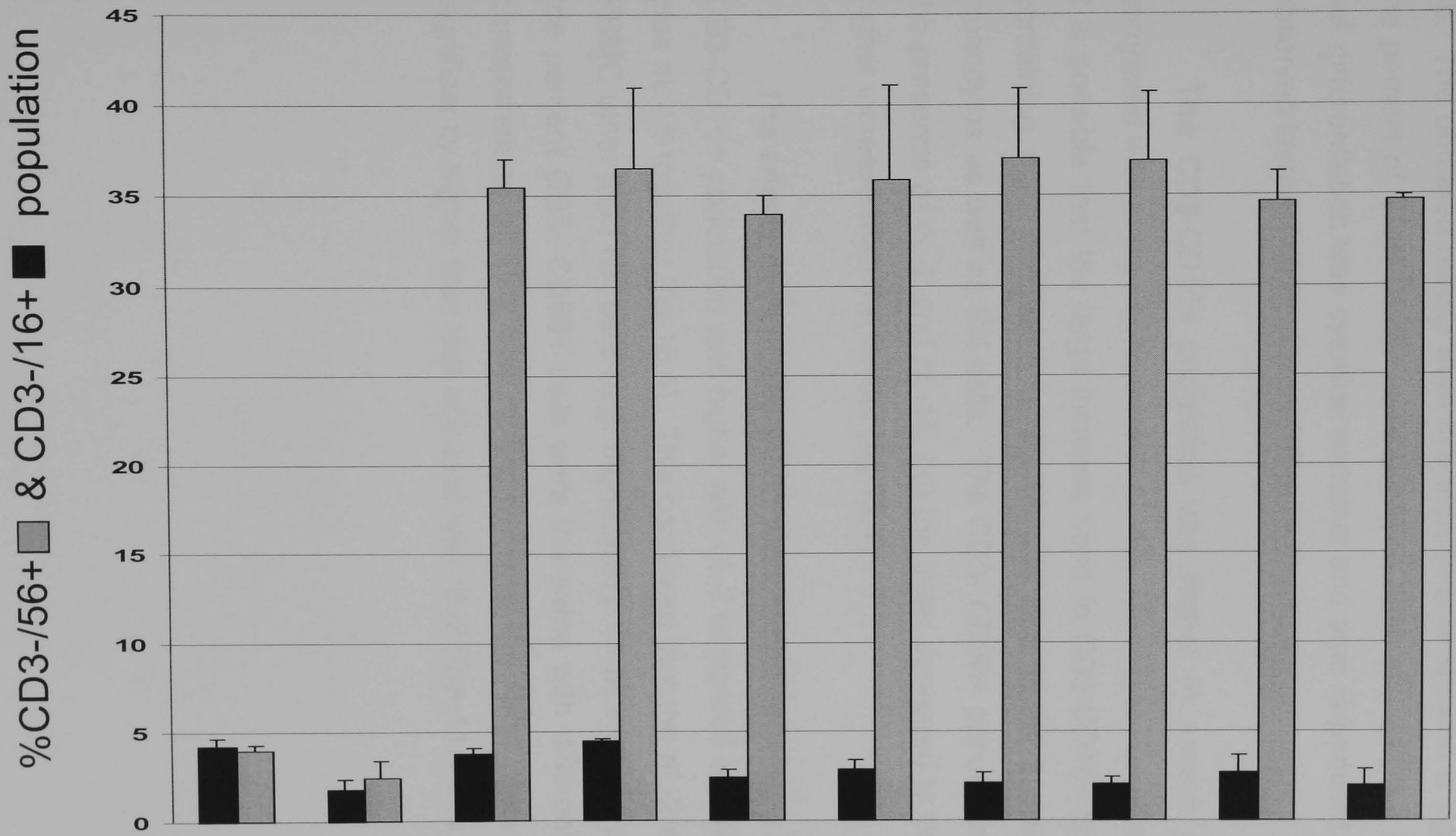
To observe the effect of freezing/thawing process on NKpf estimates, fresh PBMC and frozen/thawed PBMC were cultured from one healthy adult (AG) for five days. The NKpf (±SD) estimates were 668±134 with fresh PBMC and 630±130 with frozen/thawed PBMC. These were not significantly different ($p>0.05$) confirming that the freezing/thawing process does not significantly affect on NKpf estimate. Meanwhile, all samples including patient samples were treated by the same process during this study and frozen/thawed samples were assayed throughout.

4.3. Immunophenotyping studies in normal sample

The next step was to determine whether unique subsets of PBMC could be identified by flow cytometry that correlated with functionally mature progeny of NKp after five days of culture, and how and whether percent CD56+ cells in the CD3- population correlated with NKpf estimates.

Monoclonal antibodies specific for the human cell differentiation (CD) markers CD16, CD3 and CD56 were used for a two-colour flow cytometric analysis. Thus, the CD3- CD56+ and CD3- CD16+ populations were measured after five days of culture with rIL-2 and rIL-15. Figure-14.4 shows the mean percentage of the total CD3- population expressing CD56 and CD16 after five days of culture with different concentrations and combinations of rIL-2 and rIL-15 added on day zero and with a control containing no cytokines adding on day zero or on day five. The significance of the difference in percentage of CD3- CD16+ and CD3- CD56+ in relation to NKpf was analysed by the two-tailed student 't' test.

The obtained results with no cytokines showed that there was no change in the percentage of CD3- CD16+ and CD3- CD56+ on day zero and day five, while after adding rIL-2 or rIL-15, a significant boost in the percentage of CD3- CD56+ cells was observed ($p<0.05$) (fig-14.4).



rIL-2(Cetus units)	0 (d-0)	0 (d-5)	25	50	10	20	25	50	25	50
rIL15(ng/ml)	0 (d-0)	0 (d-5)	0	0	0	0	10	10	20	20

Figure-14.4: Mean percentage (95% CI) of total CD3- population expressing the CD3-CD56+ and CD3-CD16+ phenotypes after five days culture with no cytokine or with different combinations of rIL-2 and rIL-5 added on day zero. $p<0.05$ between CD3-CD16+ cells with rIL-2 alone and other cytokine legends. Results are the mean of five experiments with cell from AG.

In the next experiments the CD3- CD56+ populations was investigated on day Zero, two and day five after exposure to different combinations of rIL-2 and rIL-15. The obtained results from five experiments showed a significant increase in the percent of CD3- CD56+ cells between days two and five suggesting that NK cell differentiation was cytokine sensitive and time dependent consistent with the observed increase in functional NK cells (fig-15.4).

The CD3-CD16+ population was higher in assays cultured with rIL-2 compared with assays cultured with rIL-15. Monocytes are also CD3-CD16+, and it is possible that the large increase seen in CD3-CD16+ population in culture containing rIL-2 compared to rIL-15 was due to rIL-2 driven proliferation of monocytes as well as NK cells. The CD3- CD56+ population increased both in the presence of rIL-2 and rIL-15, but the latter appeared to be associated with a higher increase than the former (fig-15.4).

The effects of rIL-2 and rIL-15 were tested separately and together. The CD3-CD16+ population was higher with rIL-2 compared with rIL-15 alone, or rIL-2 plus rIL-15 together (fig-16.4). This confirmed that the rIL-2 activated subsets of PBMC other than NK cells (e.g. monocytes). In all immuno phenotyping studies the percent CD3- CD56+ cells were the same with different combinations and concentrations of rIL-2 and rIL-15, whereas the NKpf obtained with IL-15 was significantly higher than that obtained with rIL-2 (figs-11.4 and 12.4).

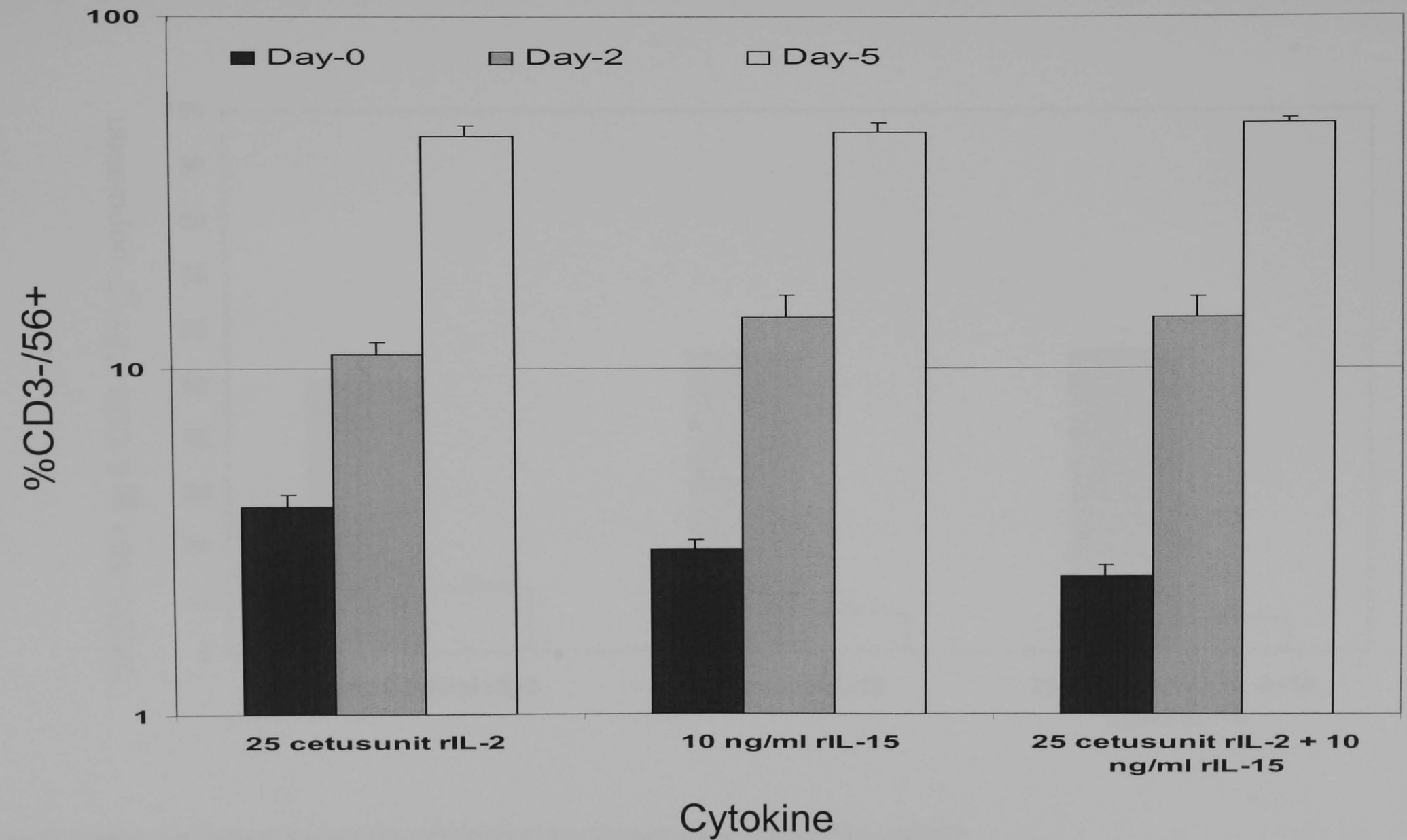


Figure-15.4: Mean percentage of total CD3- population at days 0, 2 and 5 expressing CD3-CD56+ (95% CI) plotted on a log10 scale ranging from 1-102 with different combinations of rIL-2 and rIL-5 added on day zero. $p<0.05$ between days 0&2, 0&5 , and 2&5 in all above conditions. $p>0.05$ between rIL-2 alone & rIL-15 alone, rIL-2 alone & rIL-2+rIL-15, rIL-15 only & rIL-2+rIL-15. Results are from three experiments with cells from AG.

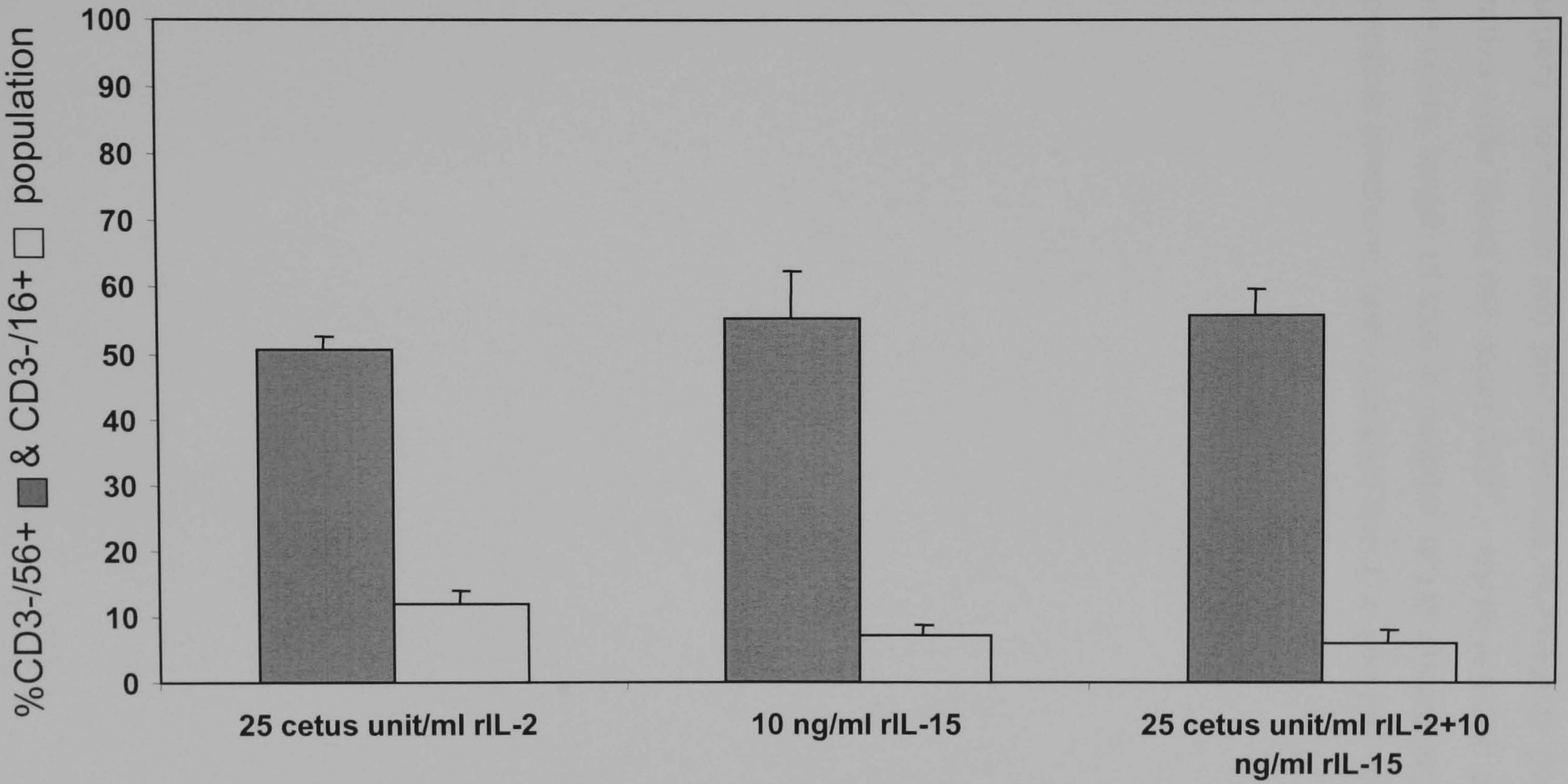


Figure-16.4: Mean percentage of total CD3- population at day five expressing CD3-CD56+ and CD3-CD16+ (95% CI) with different combinations of rIL-2 and rIL-5 added on day zero. $p<0.05$ in CD3-CD16+ cells between rIL-2 alone & rIL-15 alone, rIL-2 alone & rIL-2+rIL-15. $p>0.05$ in CD3-CD56+ cells between rIL-2 alone & rIL-15 alone, rIL-2 alone & rIL-2+rIL-15 and rIL-15 alone & rIL-2+rIL-15. The values shown are the mean of three experiments with cells from AG.

4.4.1. Patient characteristics

Table-5.4 shows patient characteristics of 120 patients including age, sex, type of surgery, admission and post operative haematocrit (Hct), admission and post operative white blood cell count (WBC), admission and post operative total lymphocyte count, length of stay in hospital, blood transfusion regimen, number of post-operative infections, post-operative infection site and cause.

Table 5.4: Characteristics of patients

Type of transfusion:	Allogeneic non-leukodepleted	Allogeneic leukodepleted	Autologous salvage	Autologous pre-deposit	No-transfused
Number of patients	8	30	40	10	32
Female: Male	6 : 2	19 : 11	20 : 20	3 : 5	17 : 15
TKR : THR	5 : 3	13 : 17	40 : 0	0 : 8	22 : 10
Age (years)	72± 8	70 ± 13	72 ± 9	57± 8	68 ± 10
Admission Hct (%)	0.37±0.05	0.38±0.05	0.40± 0.04	0.40±0.05	0.40± 0.04
Post-operative Hct (%)	0.33±0.05	0.31±0.05	0.31± 0.04	0.30±0.05	0.30± 0.04
Change	- 0.03	- 0.07	- 0.09	- 0.10	- 0.10
Admission WBC ($X10^9$/L)	7.9± 2	7.9±2	7.0±1.6	7.0±1.5	6.9± 1.6
Post-operative WBC ($X10^9$/L)	7.9± 2	9.6±2.7	8.7±1.5	8.0±1.0	8.9±2.5
Change	0	+2.0±0.7	+1.7±0.1	+1.0±0.5	+2.0±0.9
Admission lymphocyte count ($X10^9$/L)	1.90±0.5	1.50±0.4	1.83±0.6	1.63±0.5	1.70±0.5
Post-operative lymphocyte count($X10^9$/L)	1.60±0.5	1.20±0.2	1.56±0.5	1.36±0.4	1.40±0.4
Change	-0.30±0.2	-0.30±0.1	-0.27±0.1	-0.27±0.2	-0.3±0.1
Length of stay (day)	10±3.0	12± 6	11± 6	7± 1	9± 3
Number of transfused blood (unites)	2-4	1-7	0*	2	No
Number of post-op infections	1	5	4	0	4

Values are expressed as mean± SD.

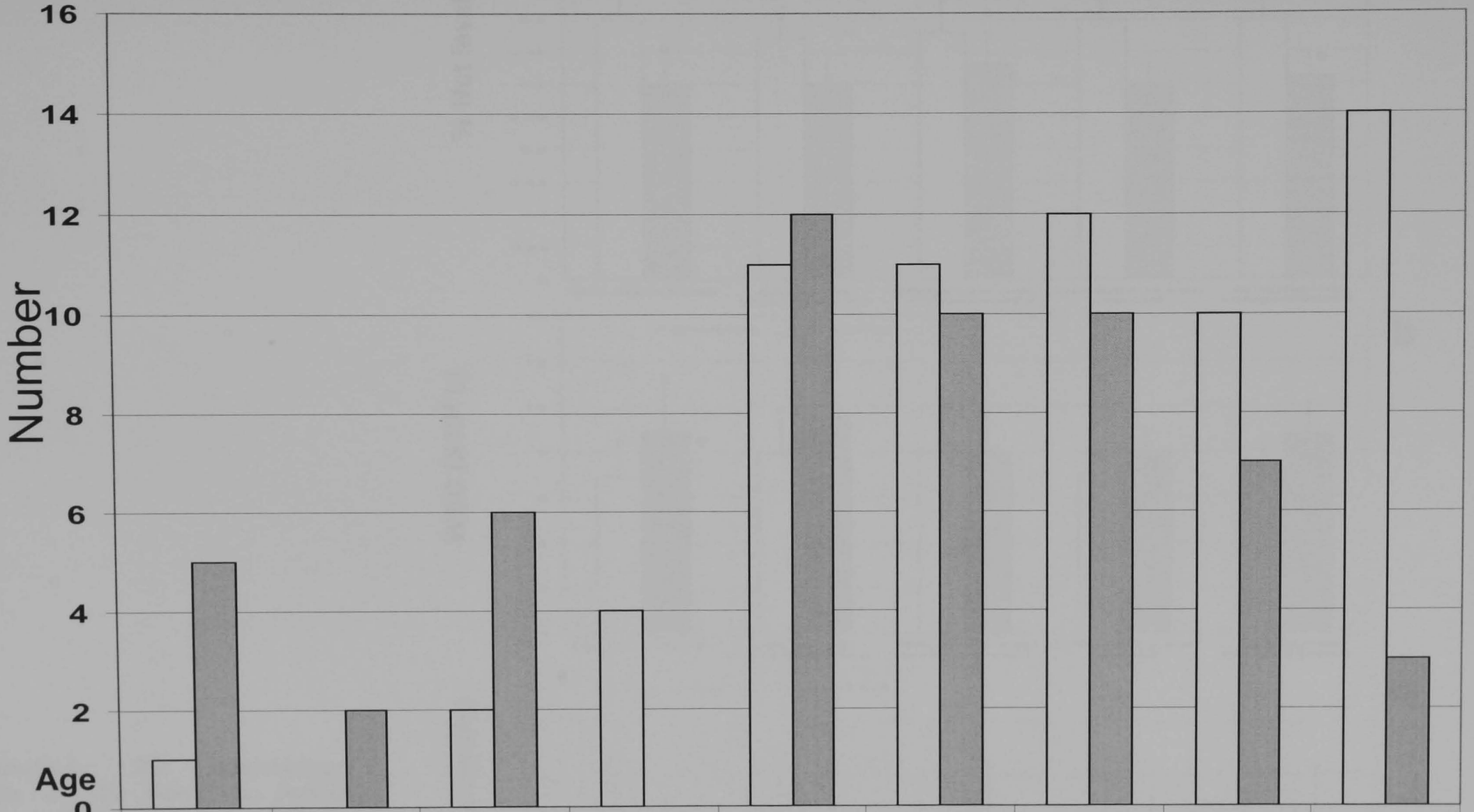
The age of patients ranged between 40 and 87 years, the mean being 70±10 years. The age range of 65 female patients was between 42 and 87 years with a mean of 72±9 years. The age of 55 male patients ranged between 40 and 84 years, with a mean of 67±11 years. There were significantly more males below 55 years and more females over 75 years (fig-17.4).

The mean age of patients who received autologous pre-deposit blood (57±8 years) was lower compared to other patient groups. There being greater motivation to travel to the transfusion centre for blood depositing in the younger fitter group.

Local policy determined that only the patients who underwent THR were eligible for pre-deposit blood transfusion programme, while only patients who underwent TKR surgery were eligible for post-operatively salvaged autologous blood programme.

Twice as many of patients underwent TKR (80) compared with THR (40). The total number of female and male patients who underwent TKR was 42 and 38 respectively. The total number of female and male patients who underwent THR was 23 and 17 respectively.

The post-operative haemotcrit declined significantly in all patient groups compared to the admission Hct level ($p<0.05$). The post-operative total WBC count rose significantly in all patient groups ($p<0.05$), except those who received allogeneic non-leukodepleted transfusion, which showed no change. By contrast, this post-operative lymphocyte count was observed to decline in all patient groups compared to the admission count (fig-18.4a-c).



	40-45	46-50	51-55	56-60	61-65	66-70	71-75	76-80	80>
□ Female	1	0	2	4	11	11	12	10	14
▨ Male	5	2	6	0	12	10	10	7	3

Figure-17.4: The number and gender of 120 study patients distributed in nine age groups.

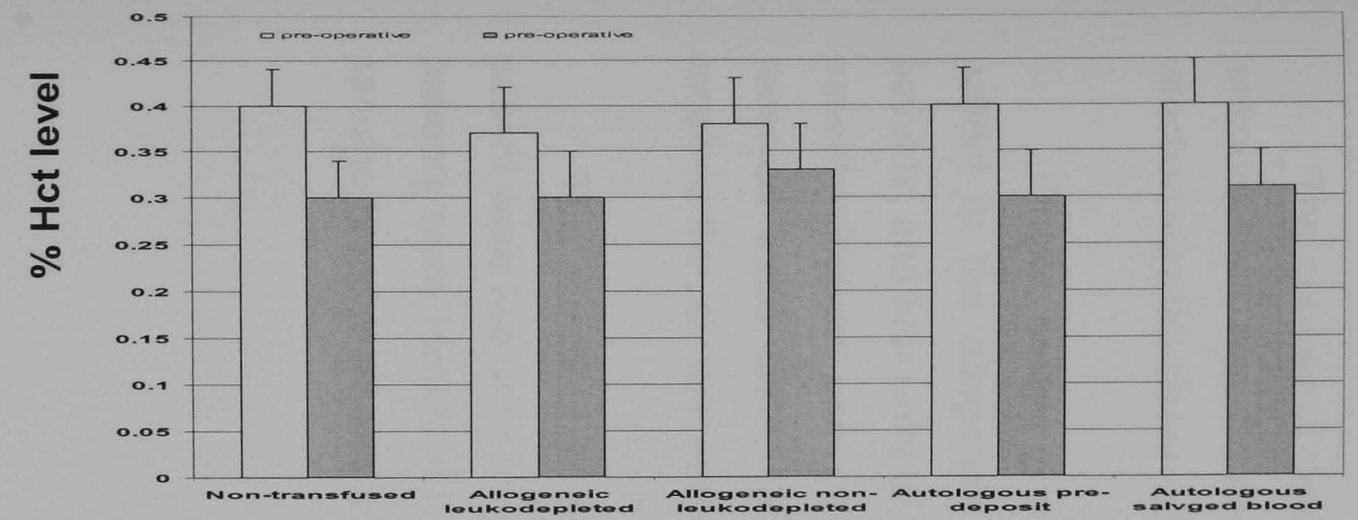


Figure-18.4: The haematology results of 120 individuals plotted before and after operation. a) Mean percentage haematocrit (Hct) level with 95% CI ($p<0.05$), b) mean total white blood cell count (WBC) with 95% CI ($p<0.05$, except in allogeneic non-leukodepleted group), c) Mean absolute number of lymphocyte with 95% CI ($p>0.05$).

Those patients receiving autologous pre-deposit blood discharged earlier than the others. In contrast, the length of stay in hospital in patents that received allogeneic leukodepleted blood was higher than other patients group.

The number of post-operative infections in those who received allogeneic leukodepleted transfusion was more than the other groups. Post-operative infection was not observed in the autologous pre-deposit transfused patients.

Finally, details of numbers of the number of units of blood given to each patient in the allogeneic non-leukodepleted group were two units to seven patients, and four units to one patient. In the allogeneic leukodepleted group one patient received one unit, thirteen patients received two units, ten patients received three units, three patients received four units and three patients received five, six and seven units, respectively.

The numbers of units of autologous pre-deposit blood given to each patient were two units to all ten patients in this group. All autologous pre-deposit patients were transfused with whole blood. Finally, the volume of autologous salvage blood reinfused averaged 650±246 mls.

4.4.2. Quantitation of NKpf before and after surgery

Figures-19.4-23.4 show the NKpf before and five days after operation in the five patient groups. In non-transfused patients and those who received allogeneic leukodepleted, allogeneic non-leukodepleted or autologous pre-deposit, the post-operative NKpf was significantly reduced ($p<0.05$). By contrast, in patients given unwashed autologous post-operatively salvaged blood, the post-operative NKpf increased significantly ($p<0.05$).

4.4.2.1. NKpf before surgery

Individual pre-operative NKpf estimates ranged from below 500 to above 3,000 NKpf/10^6 PBMC. The mean pre-operative NKpf±SD for each study group was: 1512±382 for the non-transfused group; 1397±604 for the allogeneic leukodepleted group; 1013±487 for the allogeneic non-leukodepleted group; 1447±539 for the autologous pre-deposit group and 1163±473 for the autologous salvage group. These estimates did not differ significantly between the five groups nor between gender, disease or operation types (fig-24.4). A combined mean for these groups gave a value of 1411±513.

4.4.2.2. NKpf after surgery

In contrast to the pre-operative values the mean post-operative NKpf±SD varied significantly between groups. It was 1095±415 for non-transfused group; 697±397for the allogeneic leukodepleted group; 567±187 for the allogeneic non-leukodepleted group; 757±377 for autologous pre-deposit group and 1757±477 for autologous salvage. Individual post-operative NKpf estimates varied between individuals, but in the majority of cases they retained the same rank order within their group as their corresponding pre-operative values. In four of the five groups the mean post-operative NKpf estimates were significantly lower than their pre-operative values ($p<0.05$) (figs-19.4-22.4). However, in those receiving autologous unwashed salvaged blood NKpf estimates were significantly higher than their pre-operative values ($p<0.05$) (fig-23.4).

By comparing results obtained with the different transfusion groups the question of whether or not this decrement in NKpf in those who received allogeneic blood, autologous pre-deposit blood was attributable to surgical trauma or blood transfusion or both. Thus the NKpf decrement observed in the allogeneic leukodepleted group (fig-20.4), was also observed in the allogeneic non-leukodepleted group (fig-21.4) excluding the possibility that it was wholly attributable to transfused allogeneic leukocytes. The autologous pre-deposit group further excluded allogenicity of the transfusate (fig-22.4) and the non-transfused group exonerated transfusions all together (fig-19.4). Therefore, the NKpf decrement was mainly but perhaps not entirely attributable to surgical trauma.

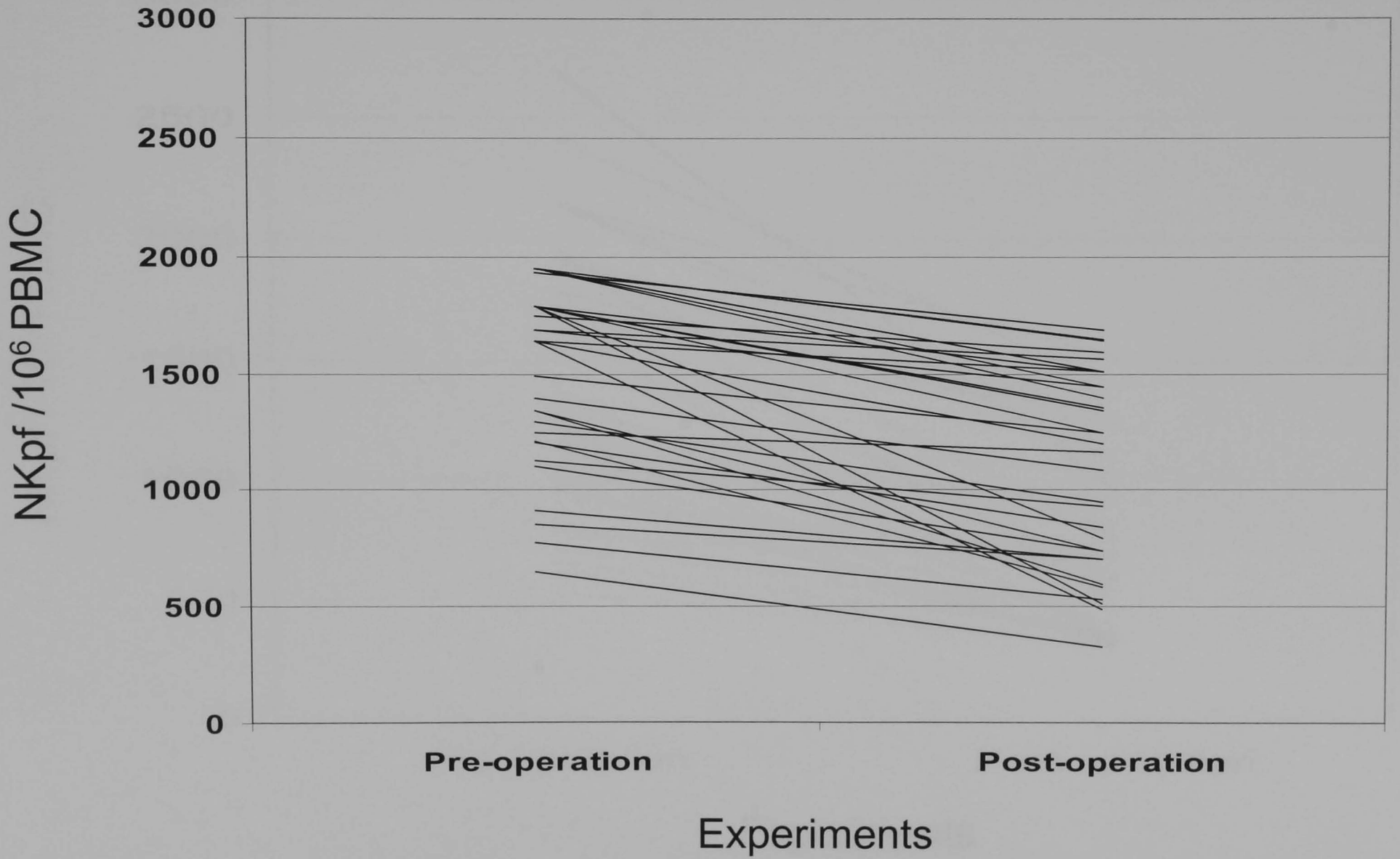


Figure-19.4: Mean NKpf (95% CI) at 5 days of culture plotted on a linear scale ranging from 0-3000/10^6PBMC before and five days after surgery in patients (32) who did not receive blood transfusion ($p<0.05$).

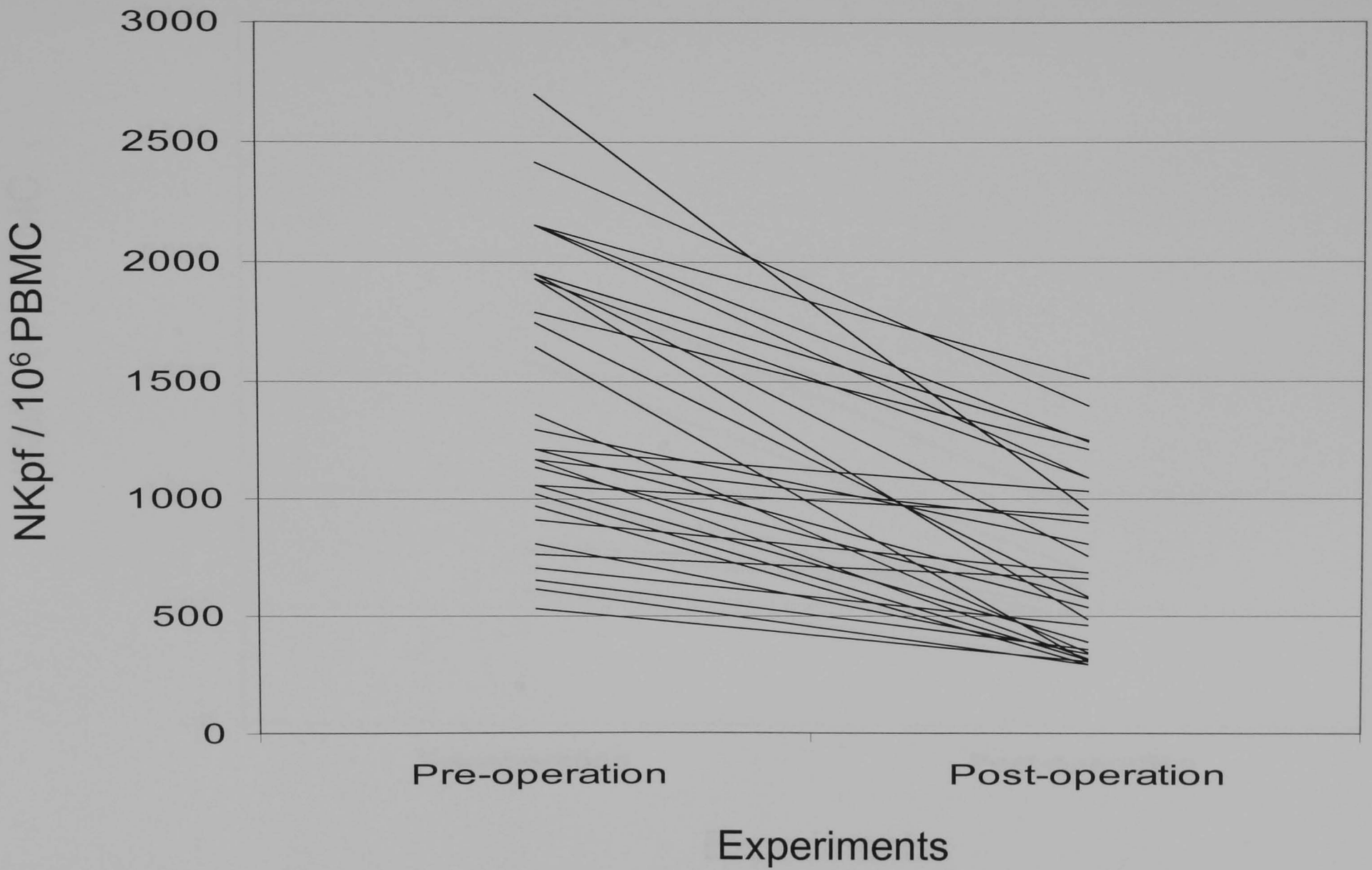


Figure-20.4: Mean NKpf (95% CI) at 5 days of culture plotted on a linear scale ranging from 0-3000 /10^6PBMC before and five days after surgery in patients (30) who were transfused with allogeneic leukodepleted blood ($p<0.05$).

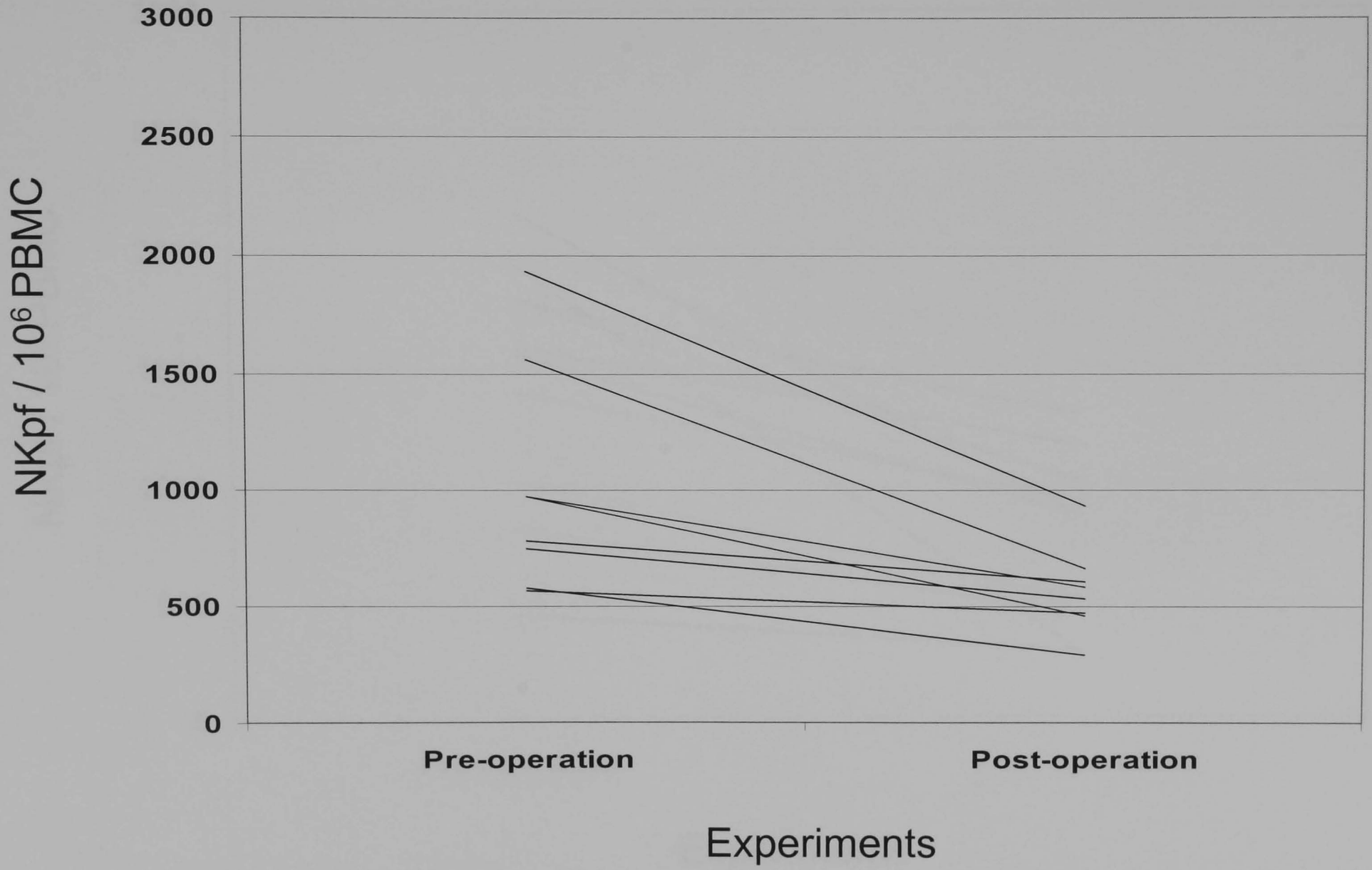


Figure-21.4: Mean NKpf (95% CI) at 5 days of culture plotted on a linear scale ranging from 0-3000 /10^6PBMC before and five days after surgery in patients (8) who were transfused with allogeneic non-leukodepleted blood ($p<0.05$).

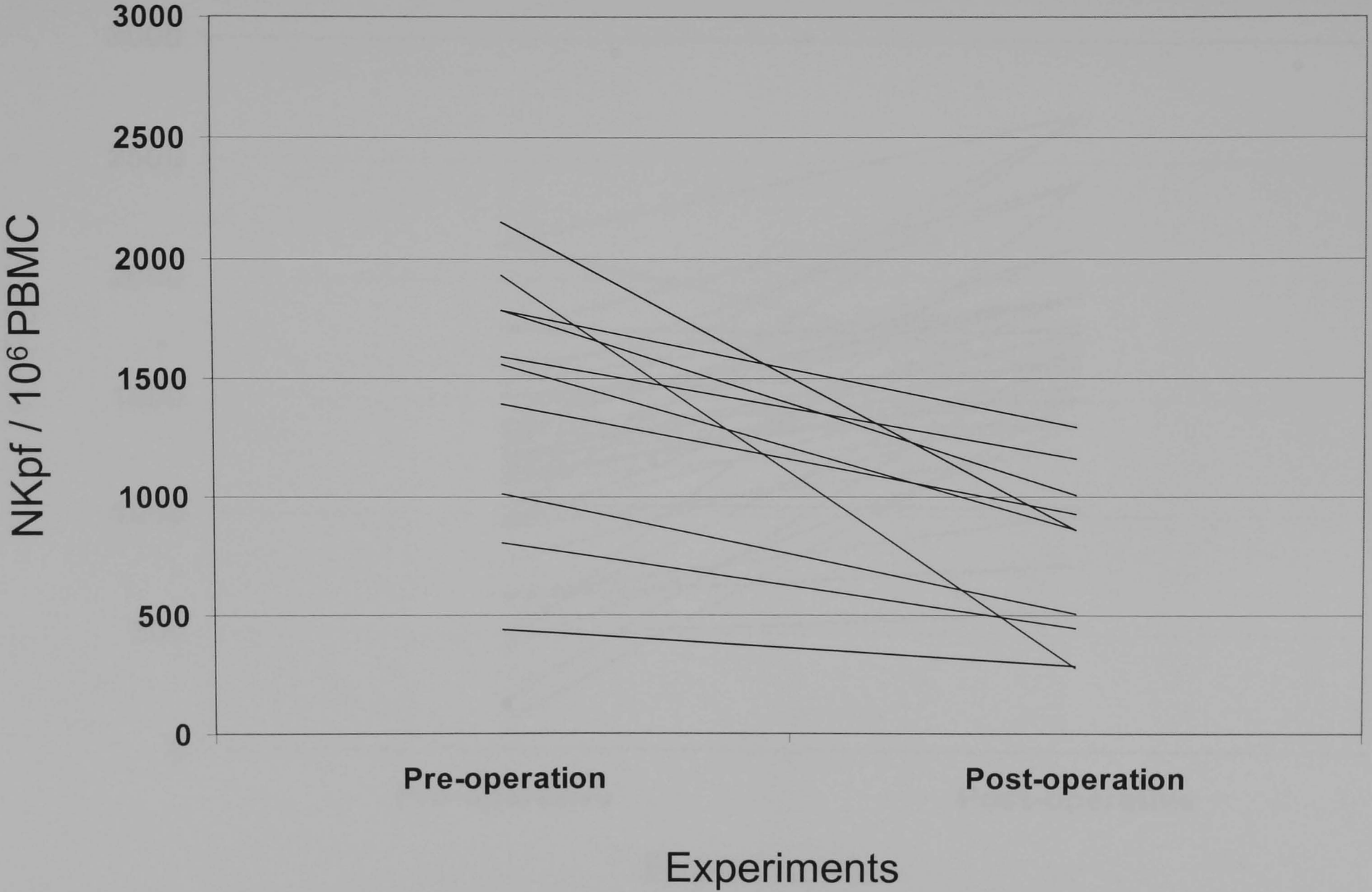


Figure-22.4: Mean NKpf (95% CI) at 5 days of culture plotted on a linear scale ranging from 0-3000/10^6PBMC before and five days after surgery in patients (10) who were transfused with autologous pre-donated blood ($p<0.05$).

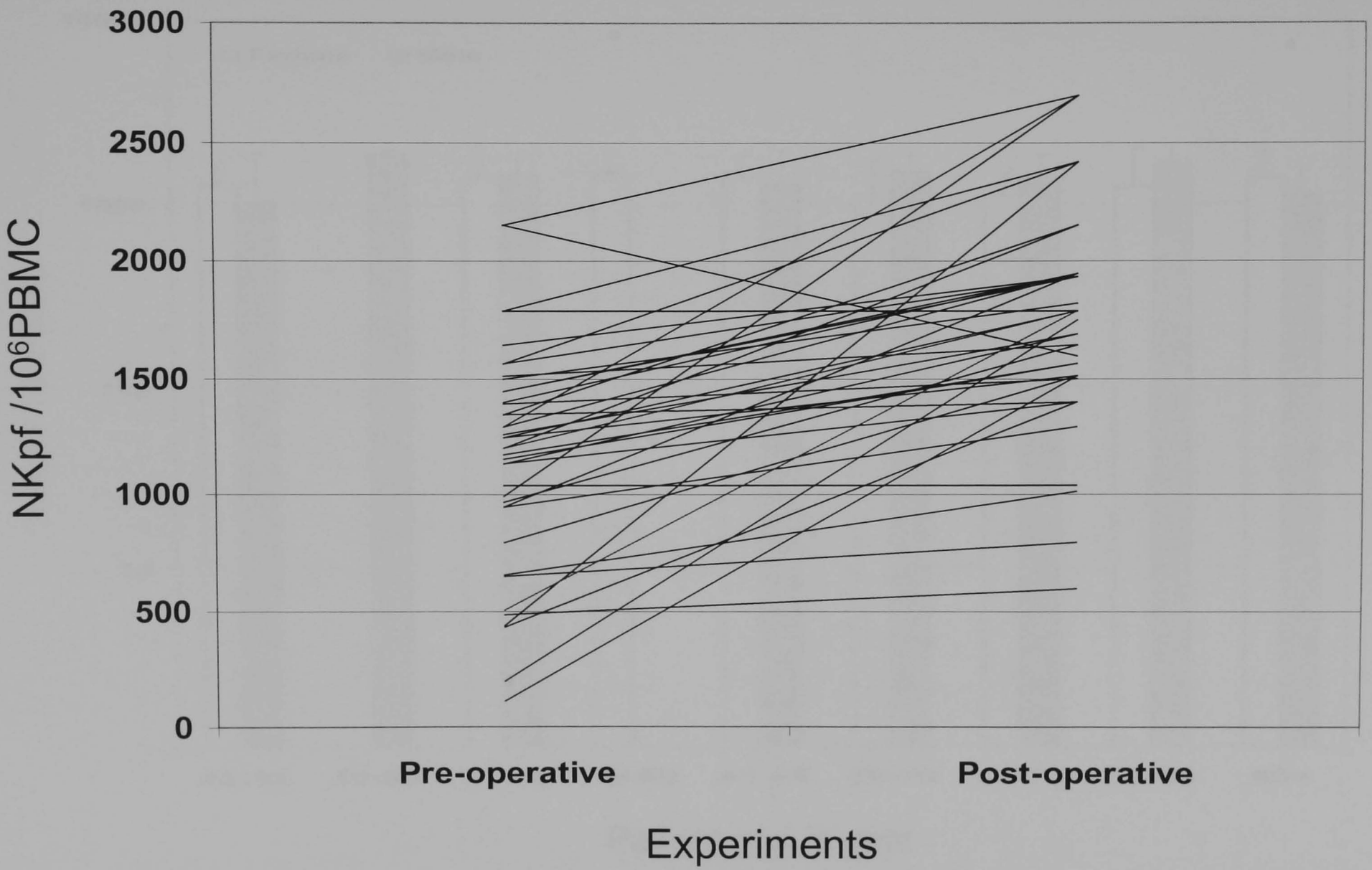


Figure-23.4: Mean NKpf (95% CI) at 5 days of culture plotted on a linear scale ranging from 0-3000/10^6PBMC before and five days after surgery in patients (40) who were transfused with autologous post-operatively salvaged blood ($p<0.05$).

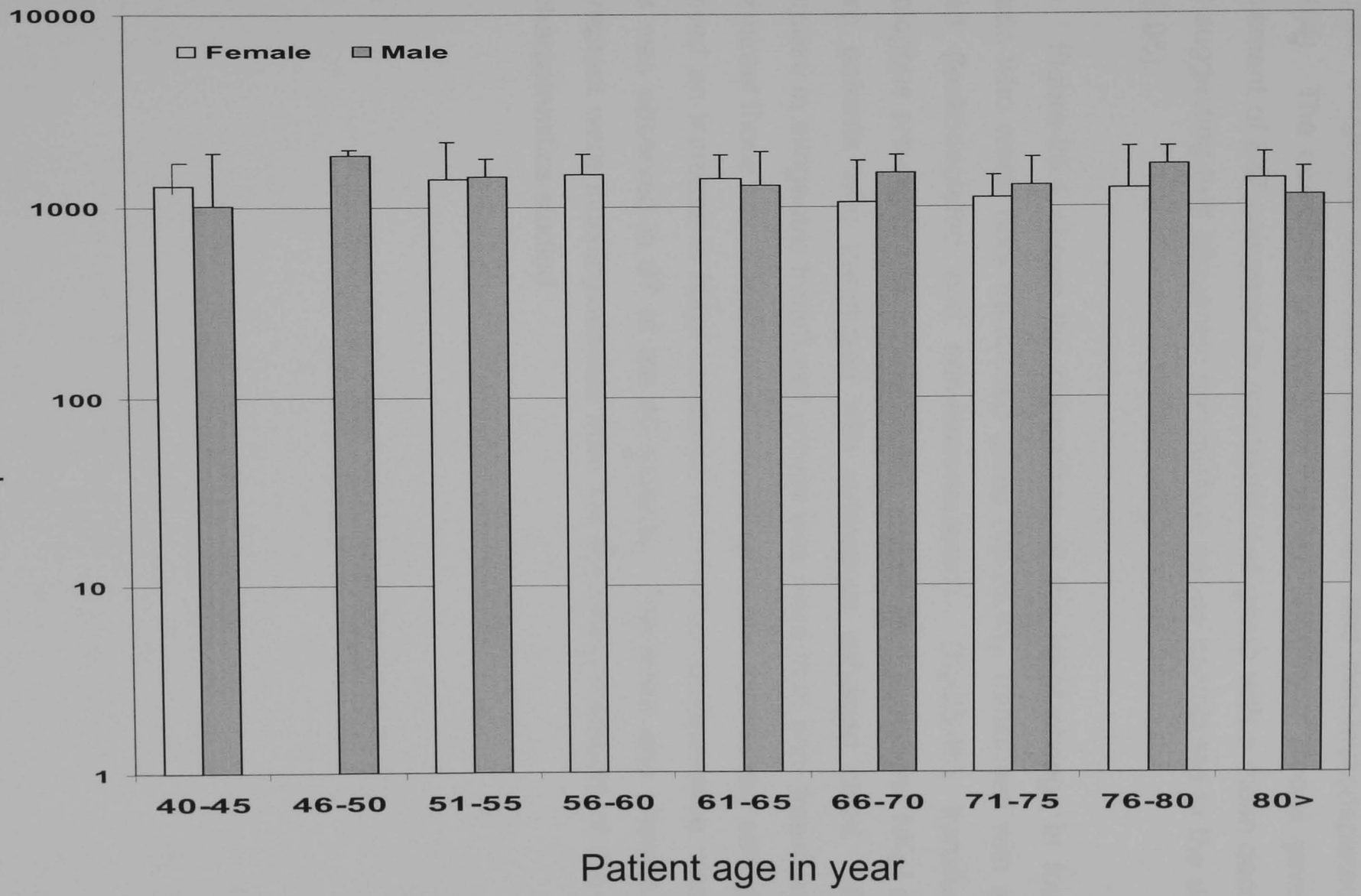
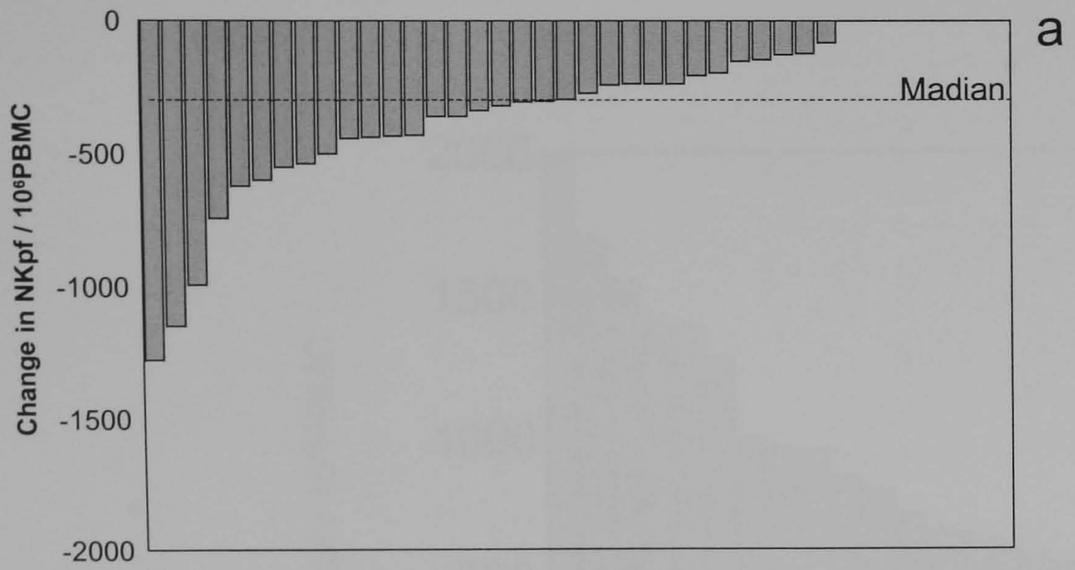


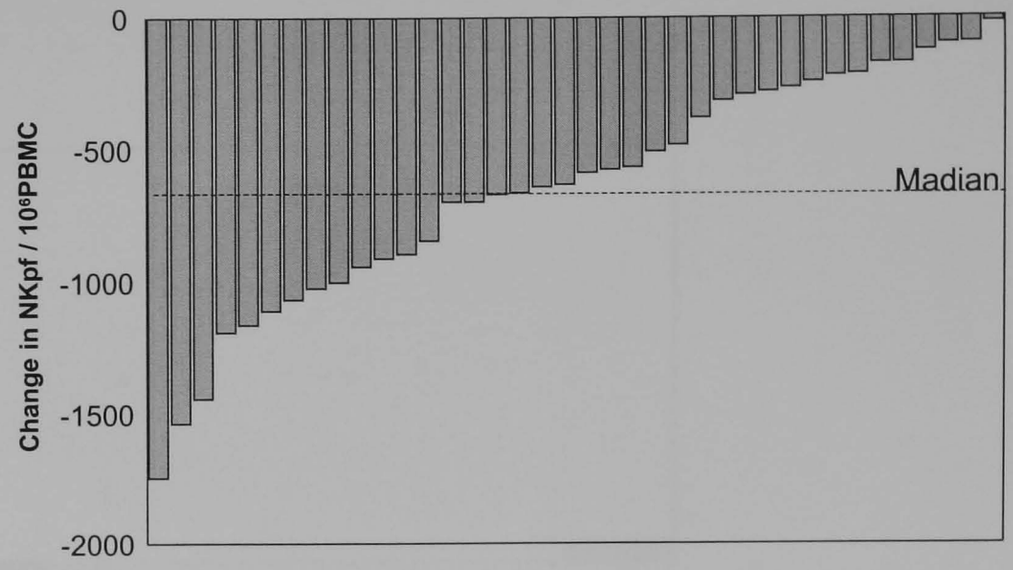
Figure-24.4: Mean NKpf (95% CI) at five days of culture plotted on a $\log_{10}$ scale ranging 1-10^4 /10^6PBMC distributed by age and gender.

A more marked decrease in NKpf was observed after allogeneic (leukodepleted and non-leukodepleted) and autologous pre-deposit transfusions compared to the non-transfused group. These estimates did not differ significantly between the two allogeneic transfused (leukodepleted and non-leukodepleted) groups ($p>0.4$). The combined groups who received allogeneic bloods gave a mean decrement of 647 compared to non-transfused group with a mean decrement of 418 suggesting that allogeneic transfusions per-se contributed to the decrement ($p<0.05$).

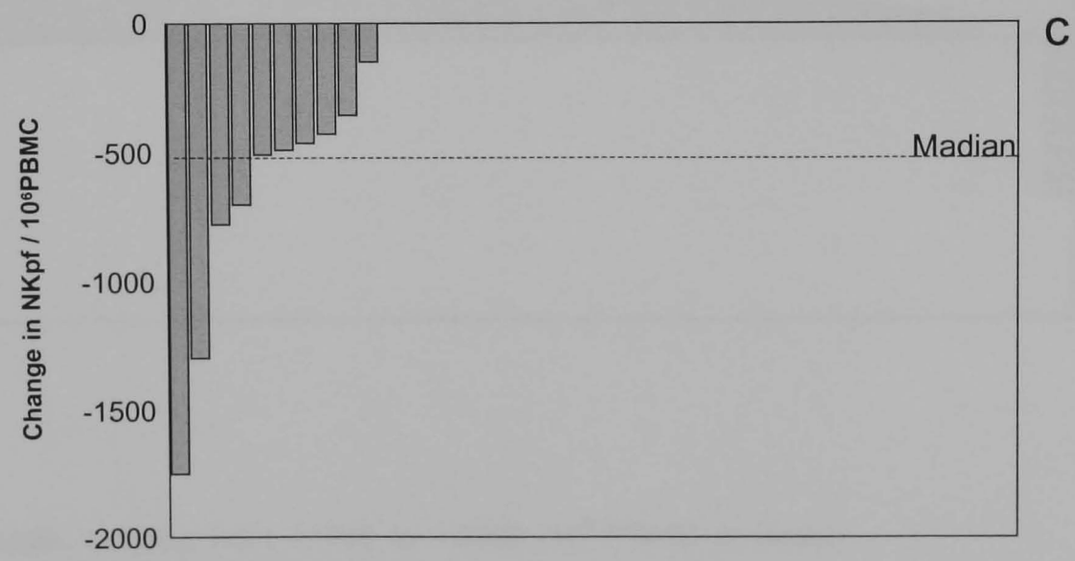
Figure-25.4 shows the comparison of the NKpf change in four patient groups who were, non- transfused group (fig-25.4a), transfused with allogeneic blood (leukodepleted and non-leukodepleted) (fig-25.4b), transfused with autologous pre-deposit blood (fig-25.4c). Figure-26.4 shows the NKpf change in those patients who transfused with autologous salvaged blood. The NKpf decrease in allogeneic transfused groups was more than non- transfused group. In contrast those who transfused with autologous post-operatively salvaged blood showed an increase in NKpf compared to individual pre-operative counterparts. This was observed in 37 of the 40 patients. The three who showed an NKpf decrement were indistinguishable from the remaining members of the group by all characteristics studied.



a



b



C

Figure-25.4: NKpf change plotted on a linear scale, ranging from –2000 to +2000 /10^6 PBMC in different patients groups. **a)** non-transfused group, **b)** the pooled of allogeneic leukodepleted and non-leukodepleted transfused groups and **c)** autologous pre-deposit group.

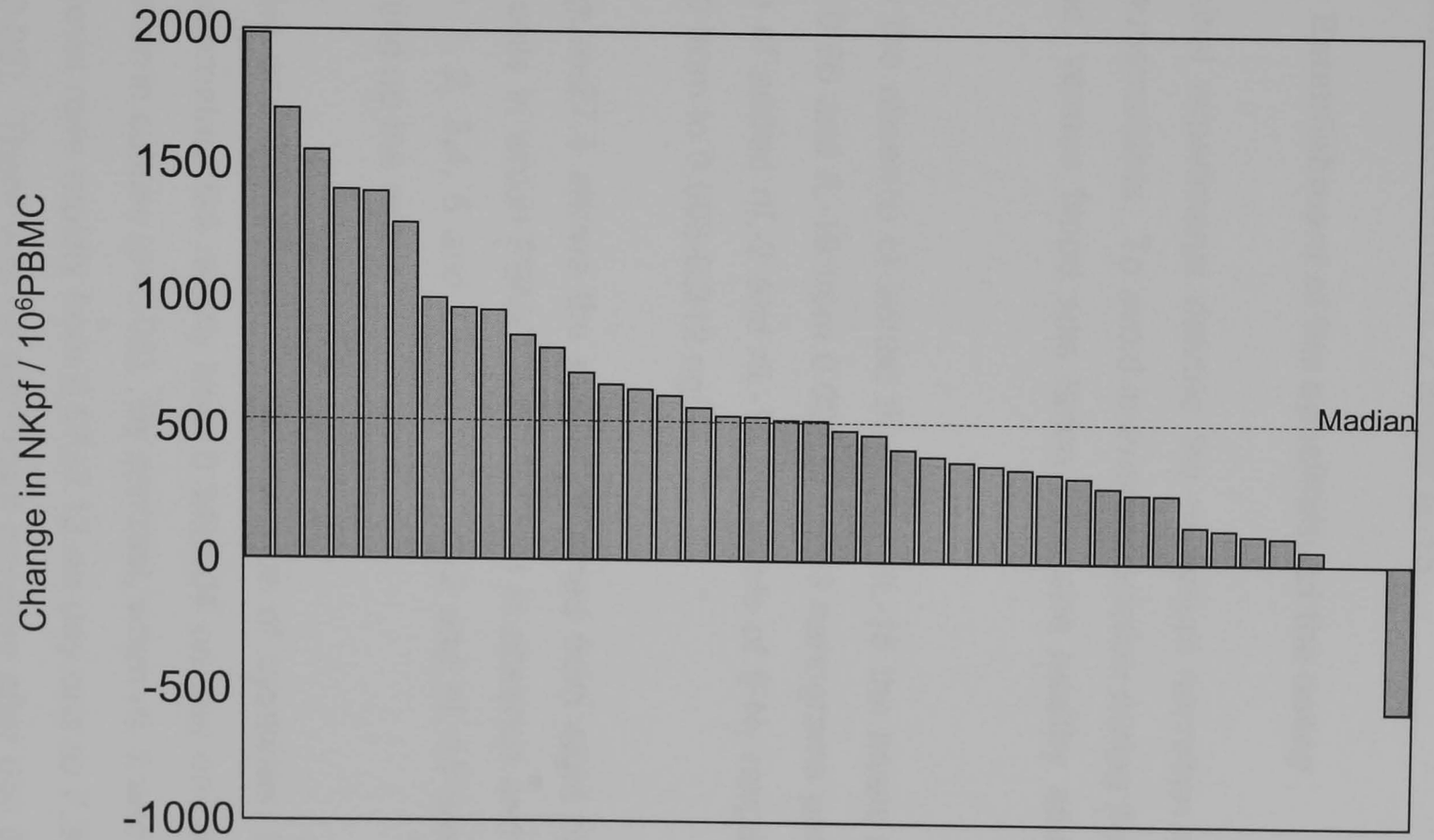


Figure-26.4: NKpf change plotted on a linear scale, ranging from –1000 to +2000 /10^6 PBMC in those patients who transfused with autologous post-operatively salvaged blood group.

4.4.3. Cytokine profiles

The production of interferon gamma (IFNγ) as an example of pro-inflammatory cytokine and interleukin 10 (IL-10) as an example of anti-inflammatory cytokine, were studied pre and post-operatively. Samples were obtained from culture supernatants at the fifth day of culture in the presence of the recombinant cytokines IL-2 and/or IL-15.

4.4.3.1. Establishment of the specificity of the assay

Initial experiments defined the maximum secretion of IFNγ and IL-10 in culture supernatants. To avoid individual variation during the development of the technique, venous blood was taken from one healthy adult (AG) at different times.

In the absence of added rIL-2 and rIL-15 the levels of IFNγ ranged from 0.016 to 0.96 and IL-10 from 0.001 to 0.003 nanograms per ml (ng/ml). In the presence of added rIL-2 and rIL-15 the levels of IFNγ ranged from 0.41 to 9.15 and IL-10 from to 0.003-0.312 ng/ml.

Figure-27.4 shows the results obtained from eight different experiments with AG cells in which PBMC were cultured in absence and presence rIL-2 and rIL-15 for 1, 2, 3,4, 5 and 7days. The rIL-2 and rIL-15 were added on day 0, when setting up the assay.

After one day culture in the absence of cytokines, the IFNγ release in culture supernatant fell rapidly from 0.34±0.04 on day one to 0.057±0.02 ng/ml on day seven in culture ($p<0.05$). By contrast, when rIL-2 and rIL-15 were added, the IFNγ level rose rapidly from 0.67±0.13 on day one to 7.36±2.2 ng/ml on day three ($p<0.05$). There was no significant increase after day three ($p>0.05$). The increase between days one and three implied rapid secretion of IFNγ in culture (fig-27.4a).

In the absence of added rIL-2 and rIL-15 the IL-10 release in culture supernatant was 3 picograms (pg/ml) on day one and it was almost unmeasurable by day two (fig-27.4b). When rIL-2 and rIL-15 were added, IL-10 levels increased rapidly from 5±0.6 on day one to 241±2 pg/ml on day five ($p<0.05$). There was no significant increase after day five ($p>0.05$). The increase between days 1-5 implied rIL-2 and rIL-15 dependent cytokine synthesis.

In summary, the results showed that cytokine driven IFNγ levels increased significantly ($p<0.05$) with duration of culture and reached a maximum plateau at day three. In all experiments IFNγ secretion levels maintained a plateau between days three and seven. Cytokine driven IL-10 levels also rose significantly ($p<0.05$) with duration of culture and reached a plateau between days five and seven. In view of this observation all ensuing cytokine assays were performed at day five.

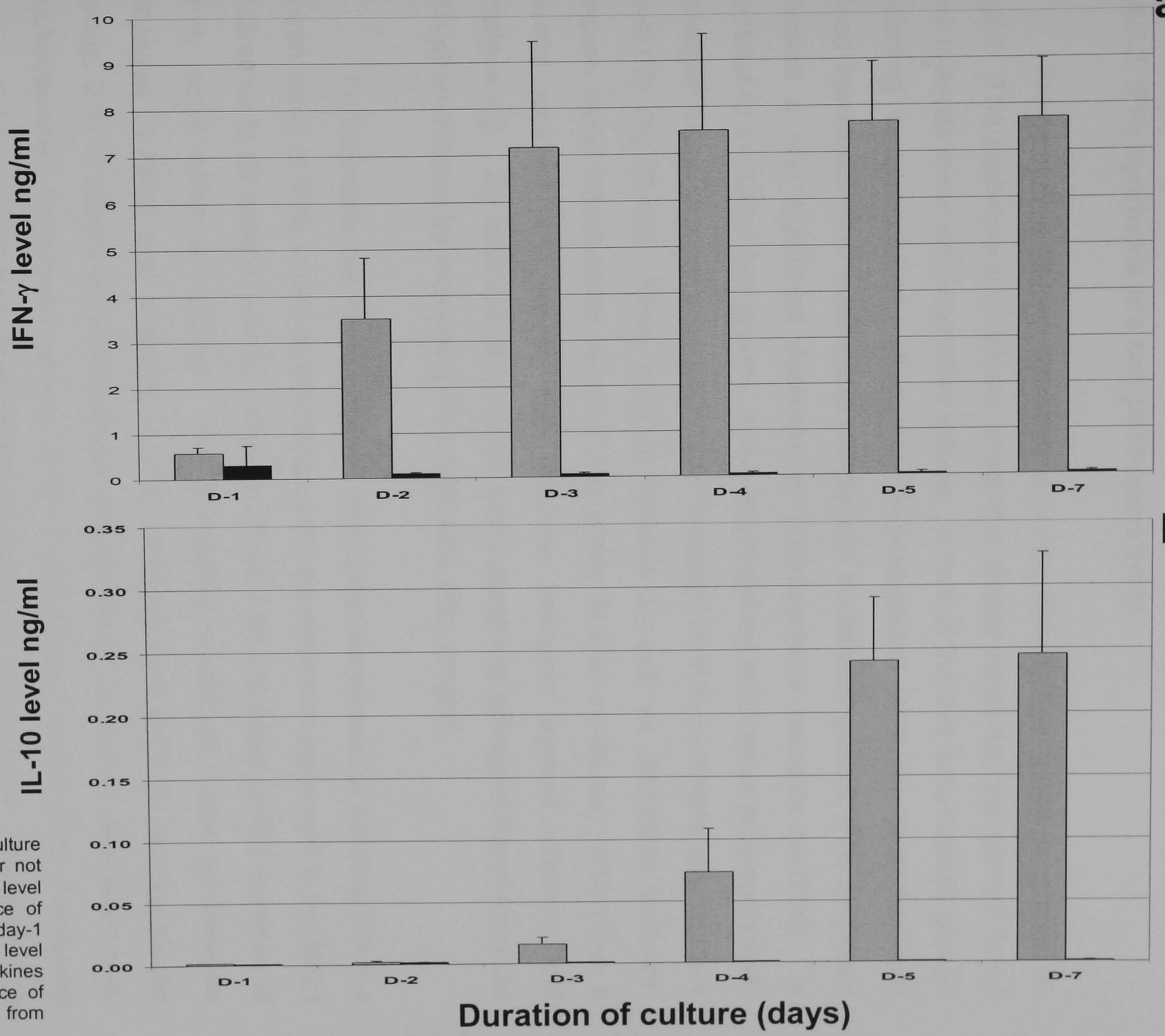


Figure-27.4: Mean cytokine synthesis (95% CI) in culture supernatant after days 1-5 and 7 after adding (grey) or not adding (black) rIL-2 plus rIL-15 on day zero. ***a)*** The IFNγ level increased between days 1&3 ($p<0.05$) in the presence of cytokines and a significant decrease was observed after day-1 in the absence of cytokines ($p<0.05$). ***b)*** The IL-10 level increased between days 1&5 in the presence of cytokines ($p<0.05$) and no increase was observed in the absence of cytokines. Results are from eight experiments with cells from AG.

4.4.3.2. IFNγ synthesis pre and post-operatively

Pre-operative values spanned a range from below 0.003 to above 50 ng/ml. The post-operative values covered a similar range but the general trend was towards lower post-operative values. In four of the five experimental groups including non-transfused, allogeneic (leukodepleted and non-leukodepleted) blood transfused and autologous pre-deposit transfused groups IFNγ synthesis showed a non significant decrease in post-operative samples ($p>0.05$). In contrast to the other four groups, those who received autologous post-operatively salvaged blood showed a non significant increase in IFNγ synthesis after surgery ($p>0.05$) (fig-28.4a). Thus, surgical traumas as well as allogeneic and pre-deposit blood transfusions suppress the ability of IFNγ synthesis. IFNγ plays a pivotal role in both innate and adaptive immunity against infection and malignancy. By contrast, autologous post-operatively salvaged blood reversed this phenomenon by increasing IFNγ synthesis after surgery.

Furthermore, the result obtained shows that decrement or increment in the mean value of IFNγ levels corresponded with decrement or increment in the NKpf estimates in all patient groups. However, there was individual patient variation of IFNγ value within all groups. The correlation coefficient value (r) between changes in NKpf and changes in IFNγ changes was 0.21 in non-transfused group, 0.31 in allogeneic leukodepleted transfused group, 0.41 in allogeneic non-leukodepleted transfused group, 0.49 in autologous pre-deposit transfused group, 0.17 in autologous post-operatively salvaged blood group. However, the correlation coefficient value (r) between NKpf and IFNγ in all patient groups combined was 0.11 (fig-28.4b). As above result shows there was a variable correlation among patient groups suggesting that stimulated NK cells were not the only source of IFNγ synthesis in culture supernatants. Although, the strongest correlation was in patients receiving allogeneic leukodepleted or autologous pre-deposit blood, this is more likely to have been affected by small sample size.

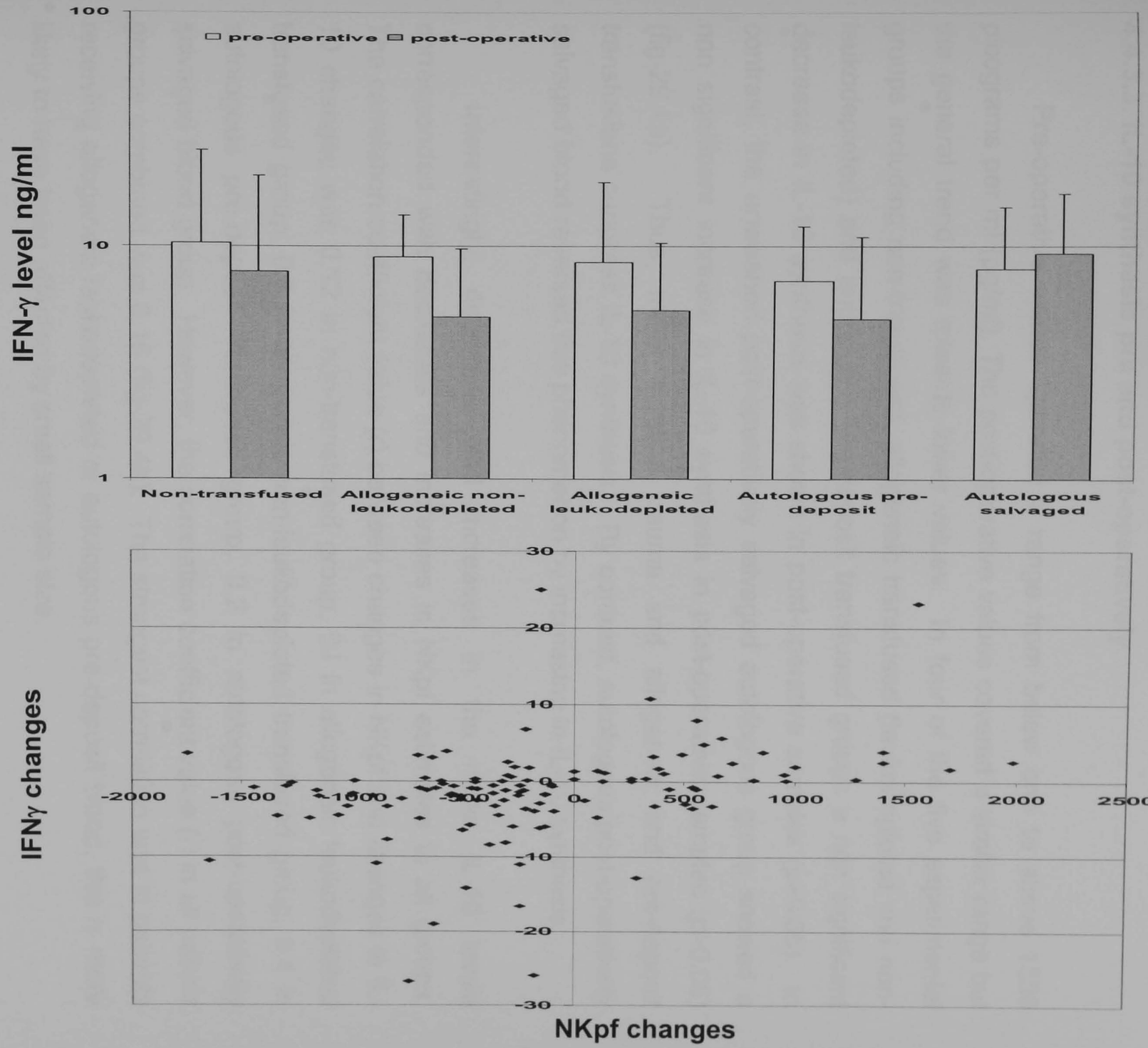


Figure-28.4: ***a)*** Pre and post-operative mean (95% CI) IFNγ synthesis at day five of culture in supernatant in patient groups. rIL-2 at 25 Cetus unit/ml plus rIL-15 at 10 ng/ml were added on day zero. ***b)*** The correlation between changes in NKpf and changes in IFNγ in all patient groups combined (r=0.11).

4.4.3.3. IL-10 synthesis pre and post-operatively

Pre-operative values spanned a range from below one to above 1500 picograms per ml (pg/ml). The post-operative values covered a similar range but the general trend was towards lower values. In four of the five experimental groups including non-transfused, allogeneic transfused (leukodepleted and non-leukodepleted) and autologous pre-deposit transfused groups a non significant decrease in IL-10 synthesis was shown in post-operative samples ($p>0.05$). In contrast, the unwashed post-operatively salvaged autologous group showed a non significant increase in IL-10 synthesis in post-operative samples ($p>0.05$) (fig-29.4a). Thus, major surgical trauma and allogeneic and pre-deposit transfusions suppress IL-10 synthesis. By contrast, autologous post-operatively salvaged blood reversed this phenomenon by increasing in IL-10 synthesis.

Interestingly, decreases and increases in the mean IL-10 levels corresponded with decreases and increases in NKpf estimates in all groups. The correlation coefficient value (r) between changes in NKpf and changes in IL-10 changes was 0.12 in non-transfused group, 0.I in allogeneic leukodepleted transfused group, 0.5 in allogeneic non-leukodepleted transfused group, 0.4 in autologous pre-deposit transfused group, 0.2 in autologous post-operatively salvaged blood group. However, the correlation coefficient value (r) in all patient groups combined was 0.16 (fig-29.4b). The strongest correlation was in patients receiving allogeneic leukodepleted or autologous pre-deposit blood, this is more likely to have been affected by small sample size.

Also, the IL-10 synthesis results showed that the mean value of IL-10 levels decreased or increased with the mean value of IFNγ levels in all patients groups, however, there was individual patient variation of IL-10 values within all patient groups.

The correlation coefficient value (r) between changes in IFNγ and changes in IL-10 changes was 0.23 in non-transfused group, 0.07 in allogeneic leukodepleted transfused group, 0.2 in allogeneic non-leukodepleted transfused

group, 0.15 in autologous pre-deposit transfused group, 0.34 in autologous post-operatively salvaged blood group.

Figure-30.4 shows the correlation between changes in IFNγ and IL-10 in all patient groups combined (r=0.13).

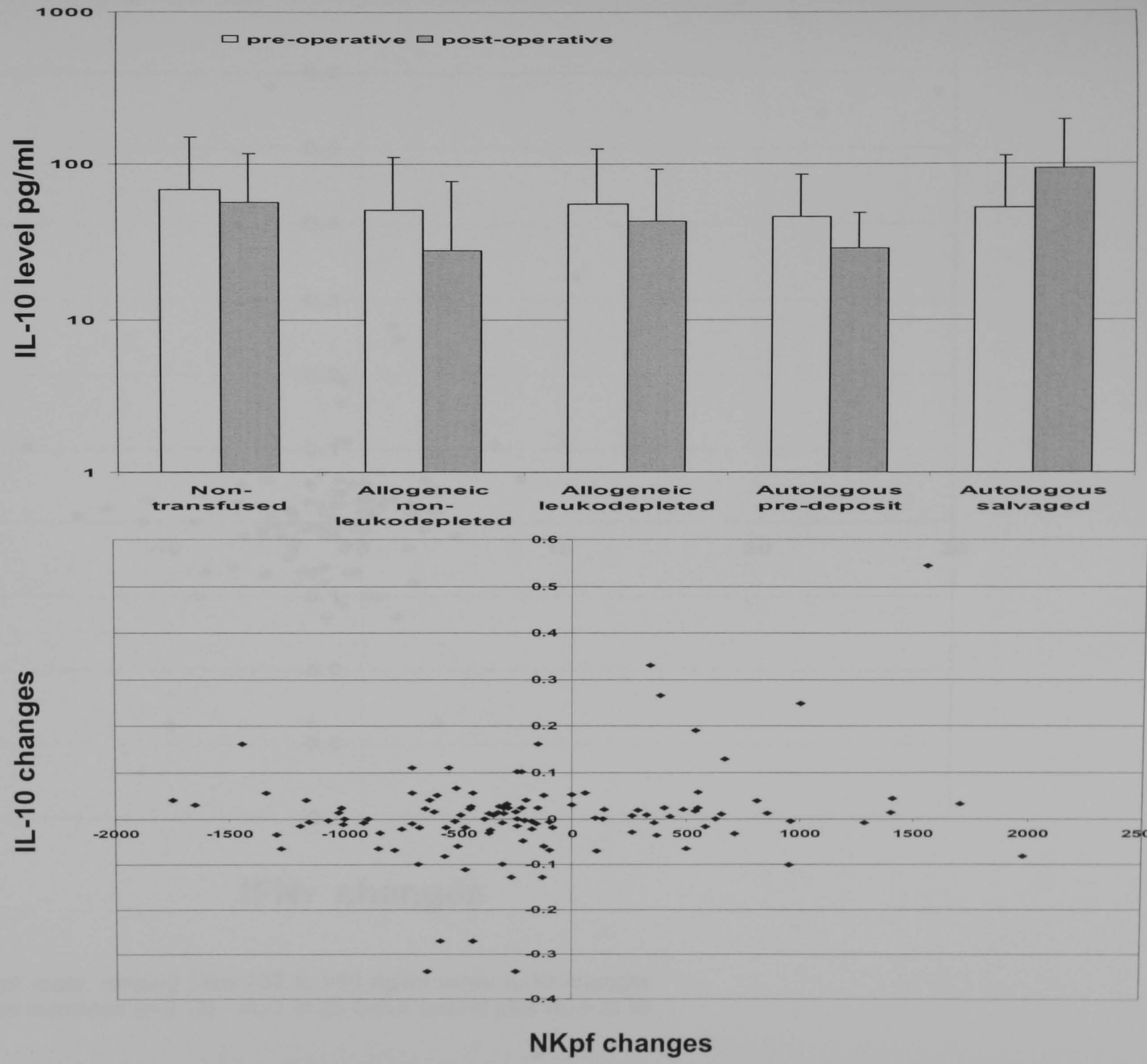


Figure-29.4: ***a)*** Pre and post-operative mean (95% CI) IL-10 synthesis at day five of culture in supernatant in patient groups. rIL-2 at 25 Cetus unit/ml plus rIL-15 at 10 ng/ml were added on day zero. ***b)*** The correlation between changes in NKpf and changes in IL-10 in all patient groups combined (r=0.16).

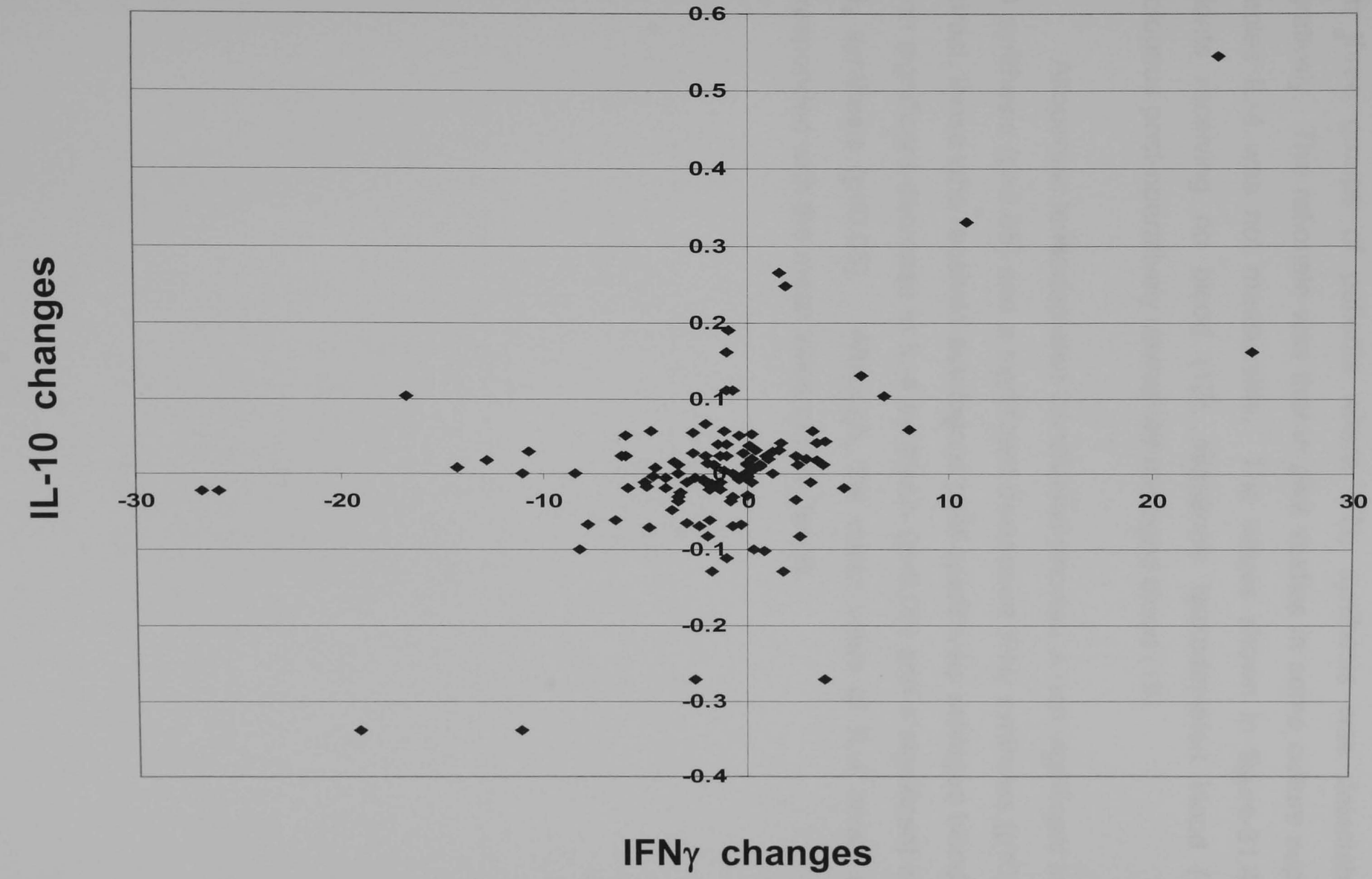
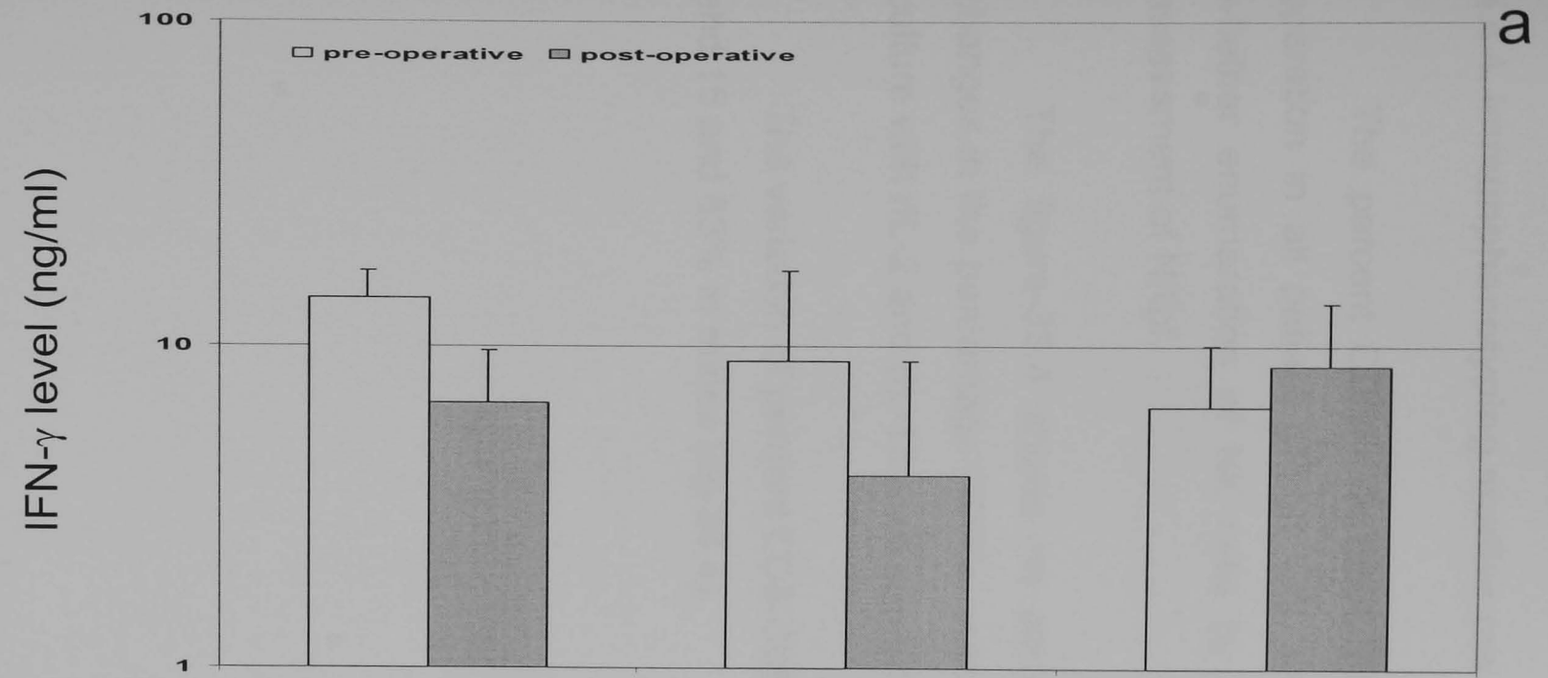


Figure-30.4: IFNγ change plotted on a linear scale, ranging from –30 to +30 ng/ml verse IL-10 changes ranging from –0.4 to +0.6 in all patient groups combined (r=0.13). rIL-2 at 25 Cetus unit/ml plus rIL-5 at 10 ng/ml were added on day zero.

4.4.3.4. IL-4 synthesis pre and post-operatively

In the next experiment the correlation between IL-4 and IFNγ synthesis was investigated. Figure-31.4 shows the results obtained from selected samples from three groups of patients where IFNγ synthesis was detectable post-operatively. The rationale was that in pilot studies in some culture supernatant samples IL-4 was not measurable. The values shown in figure-31.4 are for patients receiving no blood (12), allogeneic leukodepleted blood (10) and autologous post-operatively unwashed salvaged blood (19).

Allogeneic leukodepleted transfusion showed a non significant increased IL-4 synthesis ($p>0.05$) and a significant decreased IFNγ synthesis ($p<0.05$). By contrast, those who received autologous post-operatively salvaged blood showed a non significant decrease in IL-4 synthesis ($p>0.05$) and a significant increased IFNγ synthesis ($p<0.05$). Although, the mean value of IL-4 levels inversely corresponded with the mean value of IFNγ levels.



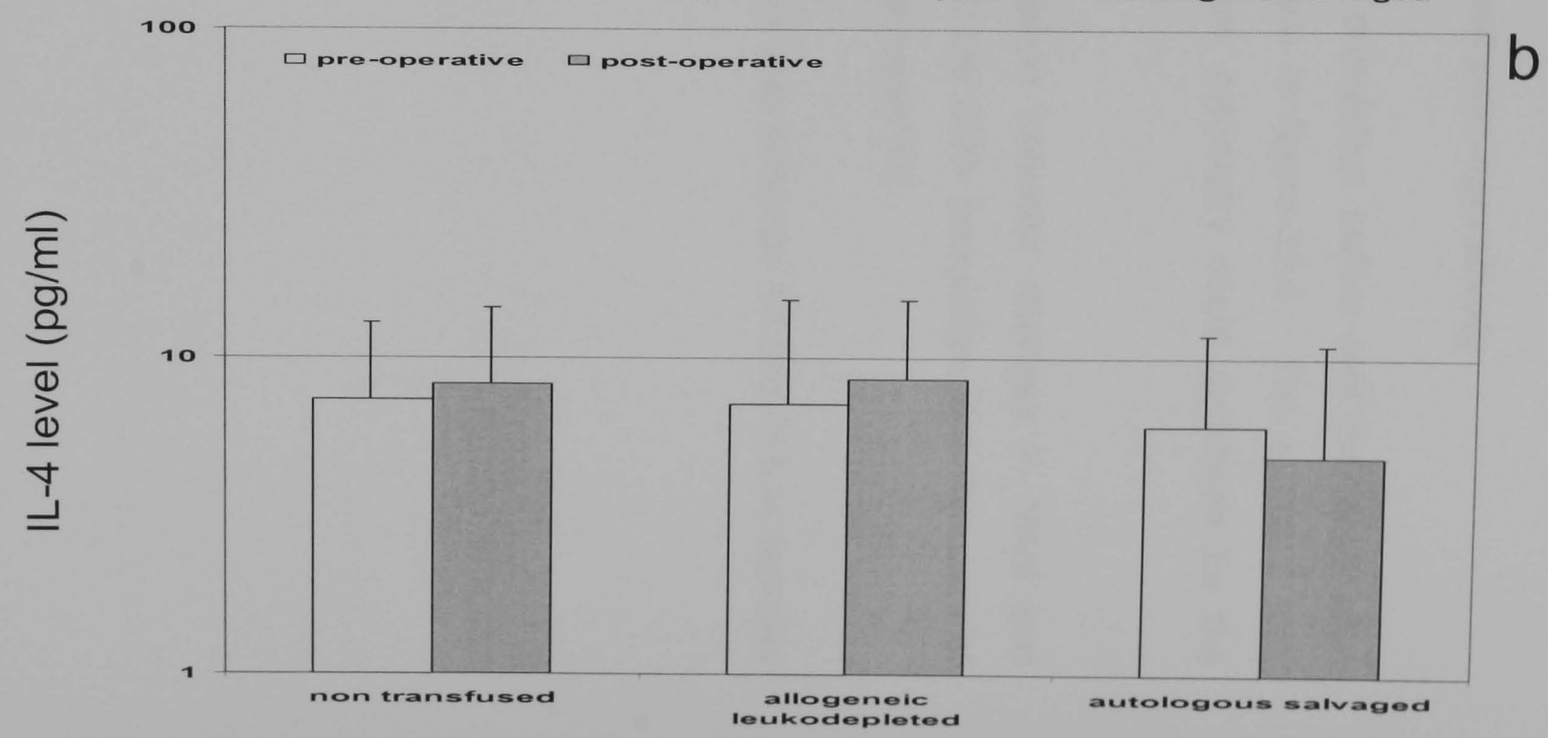


Figure-31.4: Pre and post-operative mean (95% CI) cytokine synthesis at day five of culture with rIL-2 at 25 Cetus unit/ml plus rIL-5 at 10 ng/ml added on day zero. Results are from selected patients that they were 12,10 and 19 experiments in patients receiving no blood, allogeneic leukodepleted blood and autologous post-operatively unwashed salvaged blood, respectively. ***a)*** IFNγ synthesis, $p<0.05$ in non-transfused, allogeneic leukodepleted and in autologous salvaged ***b)*** IL-4 synthesis, $p>0.05$ in non-transfused, allogeneic leukodepleted and in autologous salvaged.

4.4.4. Immunphenotyping studies pre and post-operatively

The percent CD56+ in the CD3- population before and five days after operation in all patient groups are shown in figure-32.4. The question was whether enumeration of NK cells by flow cytometry could substitute for the assessment of NKpf.

The figure-33.4 shows no correlation between changes in NKpf and changes in the percentage CD56+ cells in the CD3- population after five days of culture with rIL-2 and rIL-15 in all patient groups(r=0).

The variation of percent CD3-CD56+ was between 10 and 74% in females and 15 and 83% in males (fig-34.4).

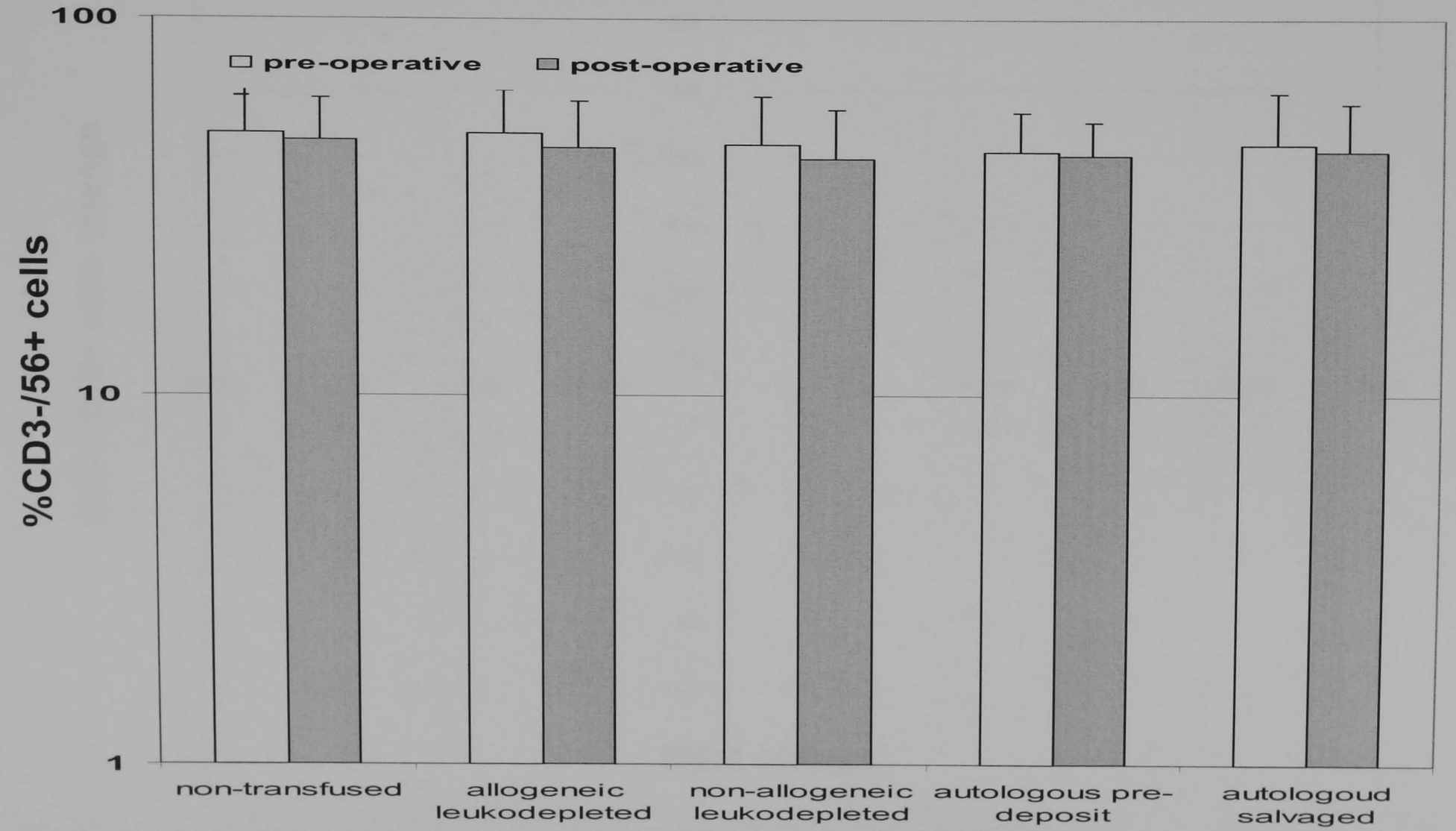


Figure-32.4: Mean percentage (95% CI) of total CD3 negative population expressing CD56 after five days culture plotted on a $\log_{10}$ scale ranging from 1-10^2 for patient groups. rIL-2 at 25 Cetus unit/ml plus rIL-5 at 10 ng/ml were added on day zero ($p>0.05$ in all patient groups).

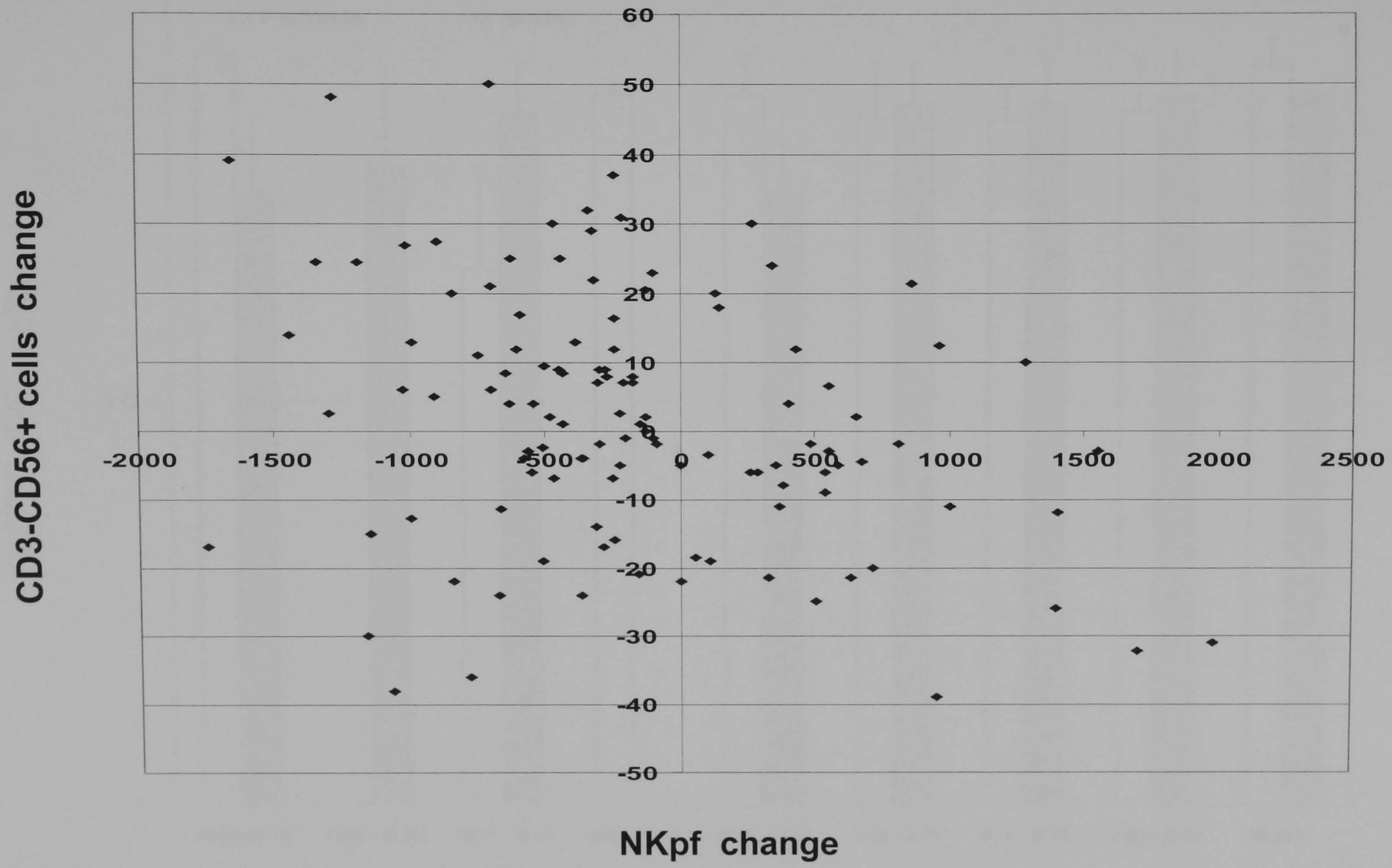


Figure-33.4: NKpf change plotted on a linear scale, ranging from –2000 to +2000 /10^6 PBMC verse CD3-CD56+ cells changes ranging from -50 to +60 in all patient groups combined (r=0). rIL-2 at 25 Cetus unit/ml plus rIL-5 at 10 ng/ml were added on day zero.

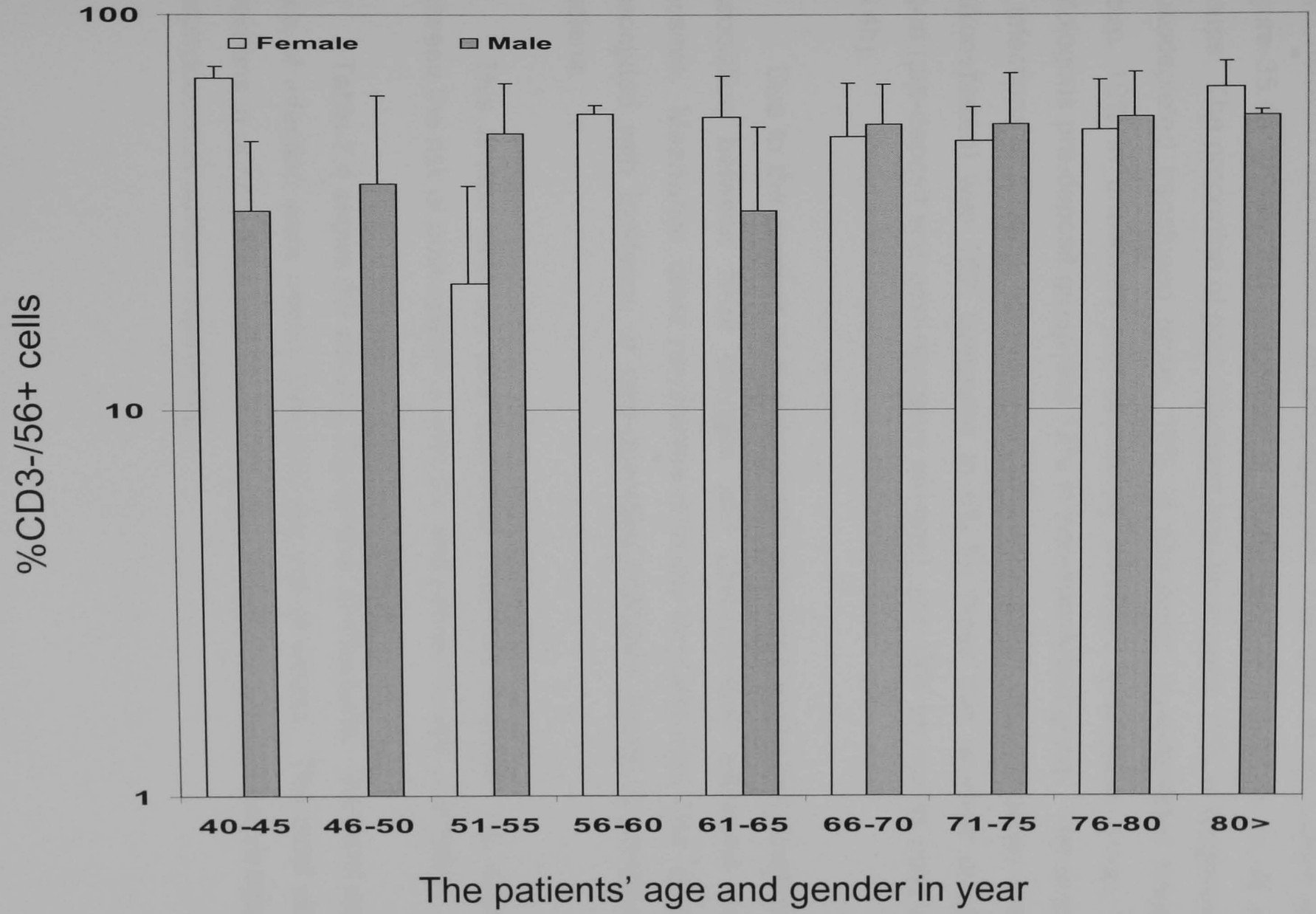


Figure-34.4: Mean percentage (95% CI) of total CD3 negative population that express CD56 plotted on a $\log_{10}$ scale ranging from 1-10^2 distributed by age and gender.

4.4.5. Post-operative infection

A total of 14 post-operative infections were identified in 14 of 120 patients in where result were available, giving an overall infection rate of 12%. Table-6.4 demonstrated the numbers of post-operative infections in all patient groups. Figure-35.4a shows the proportion of post-operative infections in all patient groups. The proportion of post-operative infections were, 12% in allogeneic non-leukodepleted transfused group, 16% in allogeneic leukodepleted transfused group, 10% in unwashed post-operatively salvaged autologous group, 0% in autologous pre-deposit group and 12% in non-transfused group. The proportion of infections in patients who received allogeneic blood (leukodepleted and non-leukodepleted) was 15% compared to 8% for those that received autologous blood (pre-deposit and post-operative salvage), and 12% for control patients (fig-35.4b).

Due to the number of post-operative infections (table-7.4), testing for an association between NKpf changes and post-operative infections was no possible. Meanwhile, other parameters of innate immunity may also have been associated with incidence of post-operative infections mainly by extra-cellular bacteria.

This limited data on post-operative infections indicated no correlation between the risk of post-operative infection and gender or type of surgery.

Table-7.4 shows the site and the number of infections. The most common sites of infection were urinary tract and the site of wound. The most common infections in urinary tract and the wound sites were with gram-negative bacilli and Staphylococcus aureus respectively.

Table-6.4: The number and the proportion of post-operative infections

Type of transfusion:	Allogeneic non-leukodepletion	Allogeneic leukodepletion	Autologous salvage	Autologous pre-deposit	No-transfusion
Number of post-operative infections	1	5	4	0	4
Proportion of post-operative infections	12	17	10	0	12

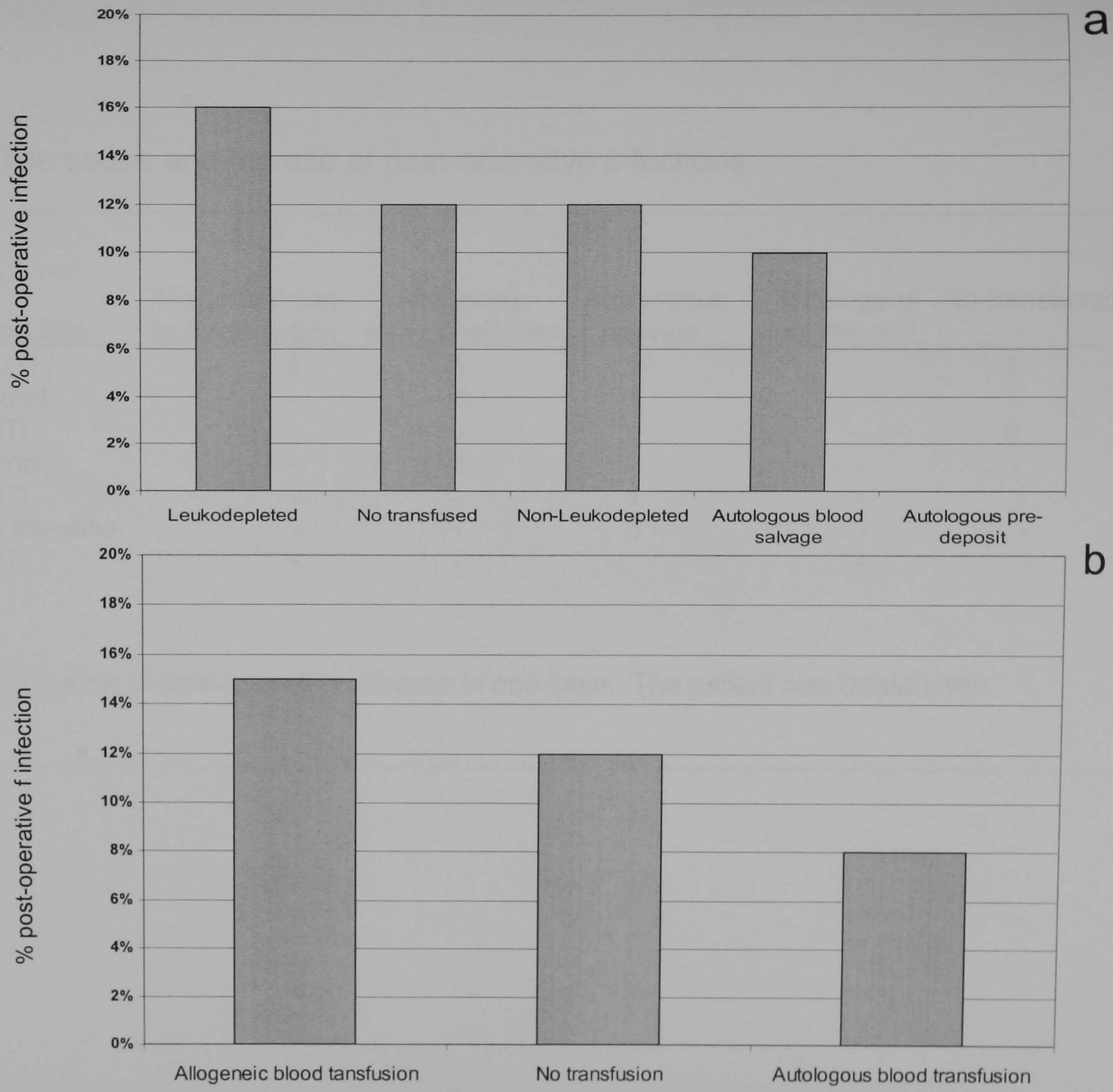


Figure-35.4: The percentage of post-operative infection in a) five patient groups and b) these patient groups including pooled allogeneic (leukodepleted and non-leukodepleted) group, non-transfused group and pooled autologous (pre-deposit and unwashed salvaged blood) group.

Table-7.4: The cause and the site of post-operative infections

Type of transfsion:	Infection Site	Allogeneic non-leukodepletion	Allogeneic leukodepletion	Autologous salvage	Autologous pre-deposit	No transfused
Coliforms	UTI	1	1	1	-	1
Staphylococcus Aureus	wound	-	1	1	-	1
Pseudomonas	UTI	-	-	-	-	2
Coagulase negative Staphylococcus*	wound	-	1	-	-	-
Escherichia coli	UTI	-	-	1	-	-
Candida	UTI & intestine	-	-	1	-	-
Entercoccus+ Escherichia coli Clostridium	UTI	-	1	-	-	-

*Coagulase negative Staphylococcus was the cause of post-operative infection in one case. The patient was treated with antibiotics.

4.4.6. Length of hospital stay

Figure-36.4 shows that autologous pre-deposit transfused patients appeared to be discharged earlier from hospital than the other groups. By contrast, the patients who received allogeneic leukodepleted blood stayed in hospital more than the other groups.

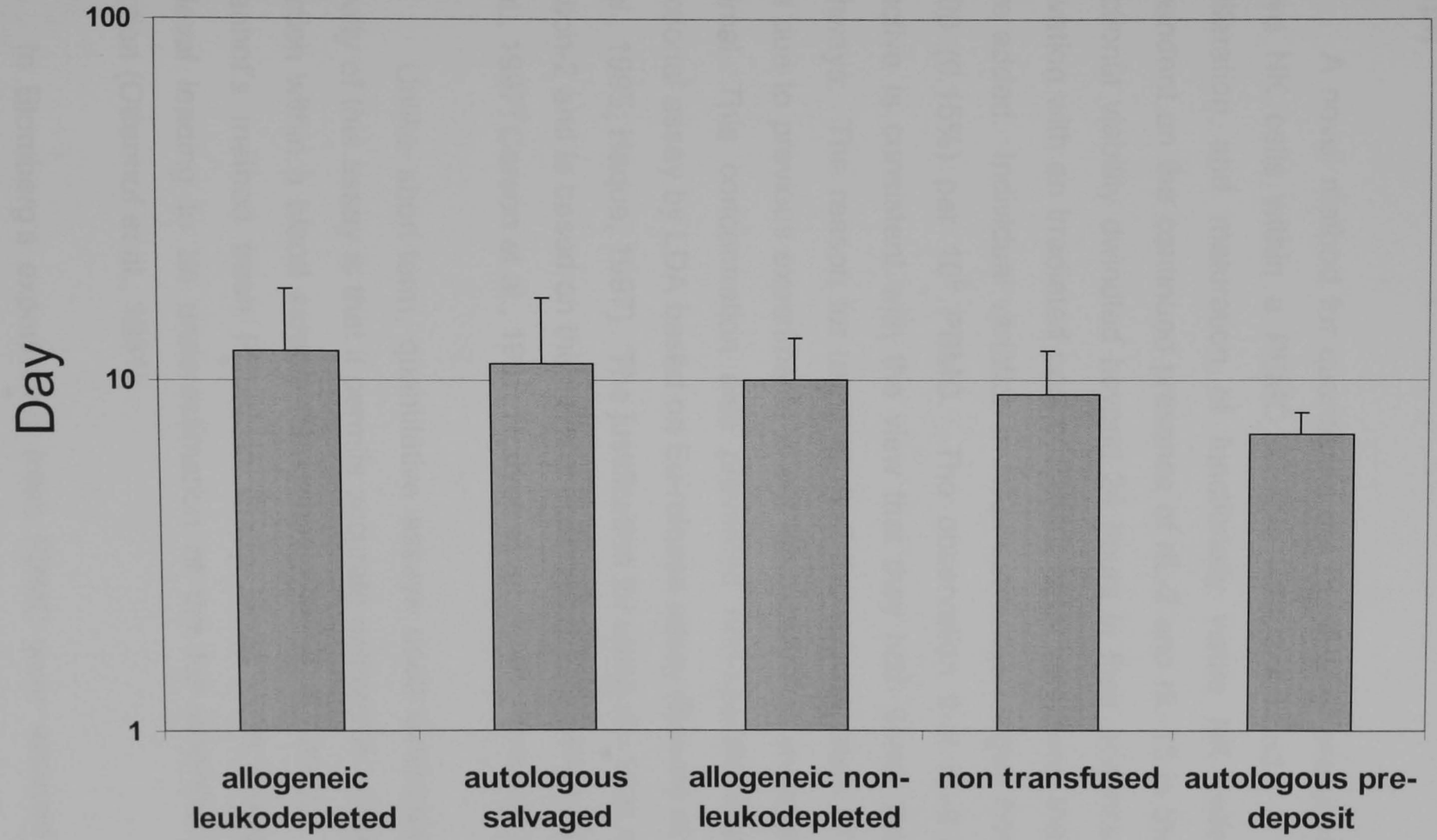


Figure-36.4: Mean length of hospital stay (95% CI) in all patient groups. Patients receiving autologous pre-deposit blood significantly ($p<0.05$) discharged earlier than the other groups.

Section 5.

Discussion

5.1. Development of an assay to measure natural killer precursor frequency (NKpf)

A novel method for quantifying the number of precursors of functionally active NK cells within a PBMC sample was described. It was shown that proliferation and maturation of functionally viable NK cells was absolutely dependent on the continued presence of rIL-2 and rIL-15 in the culture and that functional viability dwindled beyond 24 hours in their absence. Following allo-activation with an irradiated pool of PBMC, NKpf was lower than when cytokines were added. Individual variation in NKpf estimates ranged from 300 (0.03%) to 2,500 (0.15%) per 10^6 PBMC. The observation that rIL-2 and rIL-15 were effective is consistent with the view that they both share the same signalling pathways. The reason for using at rIL-2 the concentration of 25 Cetus unit/ml was due to previous experiments in our laboratory that showed that this was the optimal. This concentration also prevented non-specific reactions in cellular functional assay by LDA based on Eu-release assay (Bouma et al., 1992; Bouma et al., 1995; Haque, 1997). The justification for using rIL-15 is already outlined in section-2 and is based on the several observations (Carson et al., 1994; Allavena et al., 1997; Carson et al., 1997; Nguyen et al., 1998; Perera et al., 1999).

Unlike short-term, quantitative assays used extensively elsewhere the novelty of this assay is that it permits accurate estimation of the full potential NK function within a blood sample (Gharehbaghian et al., 2002). For example, in Ottenhof's method fresh PBMC in whole blood were assayed directly after retrieval leading to an underestimation of the full potential for NK cytotoxic function (Ottenhof et al., 1981).

In Blomberg's experiments fresh PBMC were assayed for NK cytotoxic function without pre-incubation with cytokines(Blomberg et al., 1986b; Blomberg

et al., 1986a; Blomberg et al., 1996). In Nagao's study on NK cytotoxic function fresh PBMC were also assayed directly without an activating period of culture with cytokines (Nagao et al., 1996). von-Zons cultured the PBMC samples overnight (18 hours) without cytokines for both NK cytotoxic function and lymphokine-activated killer (LAK) activity with 6000 IU of rIL-2 (von_Zons et al., 1997). Whereas, in Volgmann's study on LAK activity PBMC were cultured for 3-4 days with 1000 U/ml rIL-2 (Volgmann et al., 1989). In Bromelow's method PBMC were assayed without pre-incubation but the whole blood co-incubated with K-562 target cells for 18 hours to increase the sensitivity of the assay (Bromelow et al., 1998). However, this would still fail to account for NK cell that mature over five days in culture with rIL-2.

In this method, NKpf rose to a plateau after culturing PBMC for seven days and the increment in NKpf between days seven and ten was insignificant, suggesting all-available precursors had matured into functionally viable NK cells. In-vivo the story may vary with location and cytokine-producing micro-environment. The observed variation in NKpf between healthy individuals (pre-operatively values) reflects variables that have yet to be fully explored, such as clone size, specificity and number.

The present assay was based on a modification of the well-established non-radioactive Eu-release technique developed by Blomberg (Blomberg et al., 1986b; Blomberg et al., 1986a; Haque et al., 2001). It is relatively simple, sensitive and non-hazardous and performs well with non-adherent tumour cell targets. Released Eu is measured rapidly as a highly fluorescent chelate using a time-resolved fluorometer. In addition, Eu-EDTA is an inert chemical complex and does not bind to cellular proteins in target cells. It is also a small molecule and therefore more able to leave target cells after they are rendered permeable by exposure to perforin/granzymes produced by responder cells (Blomberg et al., 1996).

The Eu release method has also been shown to be as sensitive as the ^{51}Cr release method for measuring functional NK cells (Blomberg et al., 1986b;

Blomberg et al., 1986a; Nagao et al., 1996). In clinical studies, the Eu^{3+} release assay has been applied to measure NK activity in 68 healthy donors, 36 auto-immune patients and 21 cancer patients. The results obtained were almost identical with the ^{51}Cr release method and Eu release assay and it was concluded that the latter could be used as a method for measurement of human NK cytotoxic function of in large populations (Nagao et al., 1996). In von-Zons et al study, the comparison between Eu^{3+} release and ^{51}Cr release assays for NK and LAK function in 15 apparently healthy volunteers and 31 patients were shown to be comparable (von_Zons et al., 1997).

An advantage of this NKpf assay is that LDA are relatively insensitive to target cell concentration since the presence or absence of a culture well that is positive for Eu-release is the only criterion used to detect the presence of one or more functional NK precursor. This assumption was confirmed by showing that NKpf estimates obtained with 5,000 or 10,000 target cells per well were not significantly different. Another advantage of this method compared to ^{51}Cr release is the shortening of incubation times from 3 to 2 hours.

Both rIL-2 and rIL-15 in culture medium drove proliferation and maturation as evidenced by the plateau at day seven of culture. Although, there was a rapid and significant increase in NKp in the presence of cytokines on day 5, there was also a small increment beyond day 5 and 7 in the presence of extra cytokines added on day 5. This implied that NKp sensitisation to cytokines varied at different stages of maturation, but the majority of NKp required five days to reach maturity. When both cytokines were given together there was no increment in NKpf suggesting that the same signalling pathways were used to equal effect by both cytokines. However, in the absence of experiments with purified cell subsets, it is impossible to know whether these cytokines were operating directly through receptors on NK precursors and/or indirectly via helper T-cells in the culture. Since receptors for both cytokines shared the same β and common γ-chain they were likely to have used the same signalling pathway (Carson et al., 1997; Waldmann et al., 1998).

Human IL-15 and IL-2 ligated the β and γ sub-units of their receptors and transduce signals that cause the CD56+ subset to activate, proliferate and differentiate into functionally mature active NK cells. Gaddy and Broxmeyer showed that IL-2 and IL-15 induced functional and phenotypic maturation of cord blood NK cell subsets leading to acquisition of the CD16+56+ phenotype (Gaddy and Broxmeyer, 1997; Carayol et al., 1998). Incubation of PBMC with 10 ng/ml IL-15 for 18 h increased the antibody-dependent cellular cytotoxicity (ADCC) by three fold (Nguyen et al., 1998; Seidel et al., 1998).

Children and adults who carried the human immunodeficiency virus (HIV) were shown to have an increase in NK cytotoxic function, ADCC and the percentage of CD56+CD16+ cells when their PBMC were treated with rIL-15 for one week. This showed that IL-15 restored impairment of NK function in HIV positive patients, suggesting that autologous NK cells activated with IL-15 can be used to maximise the immuno therapeutic protocols for AIDS (Loubeau et al., 1997; Lin et al., 1998).

In-vivo, NK clonal expansion would be subject to regulatory mechanisms leading to clonal contraction by activation induced cell death or apoptosis. In-vitro, similar processes might operate explaining why the NKpf estimates following allo-activation alone were less than when cytokine was added. If, in a proportion of culture wells, apoptosis occurred this would result in lower NKpf estimates. In this allo-activation study NK cells destined for apoptosis would have been 'rescued' by rIL-2 thereby explaining the synergistic effect of the two stimuli. However, the additive effect of rIL-2 added to the allogeneic stimulus was observed as early as day one supporting an alternative view that two or more populations of cells were activated. Cells with capacity to kill K562 target cells are known to be of diverse origins. Whereas most have no T-cell characteristics others have characteristics of T-cells and are termed NKT-cells (Leite_de_Moraes and Dy, 1997; Godfrey et al., 2000).

In assays of T-cells, peptide-specific T-cell precursor frequencies measured by LDA give lower frequency estimates than when measured by

tetramer analysis (without LDA). This may be a consequence of the need for interactions between several different cell populations during T-cell maturation (e.g. T-cells and dendritic cells). Maturation and proliferation of NKp requires only the presence of soluble growth factors and may be less susceptible to this artefact. Tetramer analysis using a range of HLA Class I peptides might complement this NK functional assay by offering an insight into the distribution of inhibitory receptors, but it would be wrong to assume that all cells carrying inhibitory receptors have the potential to lyse K562 targets and vice-versa. Hence the shortcomings observed with estimates of T-cell precursors are unlikely to be applicable to the current NKpf assay.

It is suggested that the method described here gives more accurate estimates of the potential number of NKp than previous short term, single dilution assays.

5.2. Changes in NKpf after surgery and blood transfusions

In the present study NK cytotoxic function was measured in the knowledge that NK cells play a pivotal role in the first line of defence against intra-cellular micro-organisms. As such, NK cells perform a vital role in suppressing, controlling or slowing infection and neoplasia. Patients with reduced, non-functional or absent NK cells have a high frequency of infections and malignancy (Pross and Lotzova, 1993; Biron et al., 1999). Activated NK cells are able to move in solid tissues, localise to the site of metastases and kill tumour cells (Pross and Lotzova, 1993; Rabinowich et al., 1995; Okada et al., 1996). A significant correlation was shown between NK cell cytotoxic function and fewer metastases (Son et al., 1982; Schantz et al., 1987). Bruunsgaard showed a significant positive correlation between low number of NK cells and rapid progression to AIDS (Bruunsgaard et al., 1997). Thus a decrease in NK cell function could be reflected in increased post-operative infection leading to prolonged stay in hospital and poor prognosis after surgery.

The main findings of the current study were that joint replacement surgery was followed by a reduction in NKpf and allogeneic (leukodepleted and non-leukodepleted) blood transfusions added to this decrement. By contrast, transfusion with unwashed salvaged blood drained from the wound site post-operatively was not only associated with a restoration of NKpf to pre-operative levels, but in many cases to levels over and above their pre-operative values. Since these assays may reflect the integrity of innate immunity against pathological micro-organisms, they may have clinical and economic significance.

Some studies in orthopaedic surgery suggested that patients receiving allogeneic blood transfusions showed lower NK cytotoxic function associated with higher post-operative infection, longer hospital stay and higher hospital charges (Murphy et al., 1991; Triulzi et al., 1992; Heiss et al., 1997a; Blumberg and Heal, 1998; Bordin et al., 1999). Furthermore, in patients with malignancy suppression of NK cytotoxic function after allogeneic blood transfusions was associated with increased risk of tumour recurrence and decreased survival (Tartter and

Martinelli, 1987; Tartter et al., 1987; Tartter, 1988d; Tartter et al., 1989; Jensen et al., 1990; Jensen et al., 1992; Mathiesen et al., 1994; Mathiesen et al., 1998). Studies comparing immune function after laparotomy as opposed to laparoscopic surgery confirmed the association between surgical trauma and lower NK cytotoxic function (Pollock et al., 1992; Griffith et al., 1995; Bruns et al., 1996; Kutza et al., 1997; Andersen et al., 1998; Stanojevi‡_Baki‡ et al., 1999). Thus allogeneic blood transfusions given at the time of malignant and non-malignant surgeries induces immunomodulation morbidity and mortality.

By contrast, several retrospective and prospective clinical studies supported the idea that autologous blood does not induce immunomodulation. Patients transfused with autologous blood showed a better prognosis and less post-operative complications including lower post-operative infection, shorter hospital stay and lower tumour recurrence compared to those who received allogeneic transfusions (Murphy et al., 1991; Fernandez et al., 1992; Heddle et al., 1992; Mezrow et al., 1992; Howard et al., 1993; Busch et al., 1994a; Houbiers et al., 1994b; Blumberg et al., 1996; Blumberg and Heal, 1998). Autologous transfusion was also associated with reduce the demand for allogeneic blood and reduced hospital charges (Schaff et al., 1979; Blumberg et al., 1996; Duffy and Tolley, 1997; Schmidt et al., 1998).

In the majority of the above studies there was no attempt to address the mechanisms underlying immunomodulation. The results obtained in the current study suggest that surgical trauma was a greater cause of immunomodulation than allogeneic blood and that leukodepletion was unable to prevent impairment of NK cytotoxic function.

The change in NK cytotoxic function in the group receiving autologous pre-deposit blood could have been due to haemorrhage-induced immunomodulation leading to a shift of mature NK cells from the blood to the bone marrow and recruitment into the blood circulation of immature NK cells with weaker function (Ford et al., 1987; Lasek et al., 1987; Marquet et al., 1993a; Marquet et al., 1993b). However, this was an unlikely explanation in this study in view of the

lack of difference between the pre-operative NKpf values of the non-transfused (no significant haemorrhage) and the pre-deposit autologous group's (significant haemorrhage).

The NKpf decrement following surgery and allogeneic transfusions may have been accounted for either by a decreased inflow of NK precursor cells from the bone marrow into the blood or an increased outflow of NK precursor cells from the circulation into inflammatory sites. Alternatively NK precursors may have been inhibited or destroyed within the blood.

The reversal of this effect by unwashed post-operatively salvaged blood drained from the wound site may have been caused by soluble substances acquired from the damaged tissue. The mechanisms of action are not understood, but some possibilities are described below:

1) Cytokines. There are increased concentrations of IL-1α, IL-1β, IL-6 and IL-8 in the collected blood (Arnestad et al., 1994). Transfusion of unwashed salvaged blood increased IL-6 and IL-8 levels in the recipients' plasma. Whereas transfusion of washed salvaged blood, resulted in no elevation in these cytokines (Arnestad et al., 1995).

IL-6 is produced by Th_2 cells, leukocytes, fibroblasts, hepatocytes and gastric epithelial cells and acts as a proliferation and differentiation factor for B-cells, mast cells and eosinophils (Romagnani et al., 1998; Ding et al., 2000a).

IL-1α and IL-1β are produced by keratinocytes, monocytes/macrophages and B-cells. IL-1α and IL-1β play an important role in inflammatory and innate immune responses. They cause differentiation of T- and B-cells and induce IFNγ production by NK cells (Biron et al., 1999; Pan et al., 2001). They also stimulate fibroblast proliferation and chemotaxis, leukocyte chemotaxis and keratinocyte migration. They can act synergistically with TNFα. Moreover, IL-1 production by fibroblasts is induced by TGF-β (Schaffer and Barbul, 1998; Gharaee_Kermani and Phan, 2001).

TNFα is produced by monocytes/macrophages, activated NK and T-cells. It stimulates macrophage function, fibroblast proliferation, IL-1 production and degradation of extra-cellular matrix components by fibroblasts (Schaffer and Barbul, 1998; Gharaee_Kermani and Phan, 2001).

IL-8 is a pro-inflammatory cytokine produced by monocytes/macrophages and NK cells and acts as chemokine (Lanier, 2000a; Maghazachi, 2000). IL-8 is a member of the CXC chemokine subfamily and is chemotactic for neutrophils, monocytes, lymphocytes and NK cells (Maghazachi, 2000). Other cytokines that can recruit NK cells from bone marrow to blood circulation are IL-2, TNFα, GM-CSF, IFNγ, LTα/TNFβ (Ito et al., 1999; Glas et al., 2000).

2) Chemokines. Chemokines (chemotactic cytokines) are low molecule-weight signalling proteins that recruit various types of cells into inflammatory sites. They are divided into four subfamilies including CXC (α), CC (β), C (γ) and CX_3C (δ) (Maghazachi, 2000; Inngjerdingen et al., 2001). The CC family is chemotactic for monocytes, neutrophils and lymphocytes. Lymphotactin (Ltn) and fractakline (neurotactin) are members of C and CX_3C subfamily, respectively that induce the migration of NK cells (Maghazachi, 2000). It was shown that macrophage inflammatory protein-1α (MIP-1α), the factor termed, "regulated upon activation normal T-cell expressed and secreted" (RANTES), macrophage chemotactic protein- 1,2,3 (MCP-1,2,3), interferon-induced protein-10 (IP-10) and MIP-1β are chemotactic for NK cells (Biron et al., 1999; Glas et al., 2000).

Inngjerdingen showed that NK cells express the receptors for monokine induced by interferon (MIG), MIP-3β and IL-8. NK cells also express CCR4-the receptor for stromal-derived factor-1 (SDF-1), CCR8-the receptor for I-309, and the receptor for growth related oncogene-α (GRO-α) (Inngjerdingen et al., 2001).

Campbell showed that CD56+CD16+ NK cells migrate in response to IL-8 (Campbell et al., 2001). Otto showed an increase in expression of MIP-1α and its receptor CCR5 on NK cells after closed head injury in mice (Otto et al., 2001). Production of MCP-1 by epithelial cells is stimulated by IL-1β, TNFα and IL-6

(Biswas et al., 1998). DiPietro showed that MIP-1α produced at the site of injury strongly recruits macrophages to the injury site (DiPietro et al., 1998).

Cosman reported that NK cells produced I-309, a ligand for CCR8 that recruited and activated NK cells and macrophages (Cosman et al., 2001).

In Perera's study it was shown that IL-15 was a potent inducer of CC, CXC, and C chemokines subfamilies in lymphocytes. It also enhanced the secretion of MIP-1α and RANTES, and induced the expression of CC chemokine receptors (Perera et al., 1999).

Dinarello showed that IL-18 stimulated both CXC and CC subfamilies of chemokine. IL-18 is a pro-inflammatory cytokine producing by monocytes/macrophages and keratinocytes and its production is stimulated by IL-1, TNFα, LPS, and exotoxins from gram-positive bacteria (Dinarello, 1999).

Interestingly, Moore reported that Iodine released from the Iodosorb wound dressing recruited macrophages to the wound site resulting in increased TNFα synthesis, decrease in IL-6 synthesis and an influx of monocytes and T-cells into the site of wound (Moore et al., 1997).

Table-8.5 shows those chemokine that can stimulate NK cell proliferation, recruitment and activation.

Table-8.5: Chemokines acting on NK cells

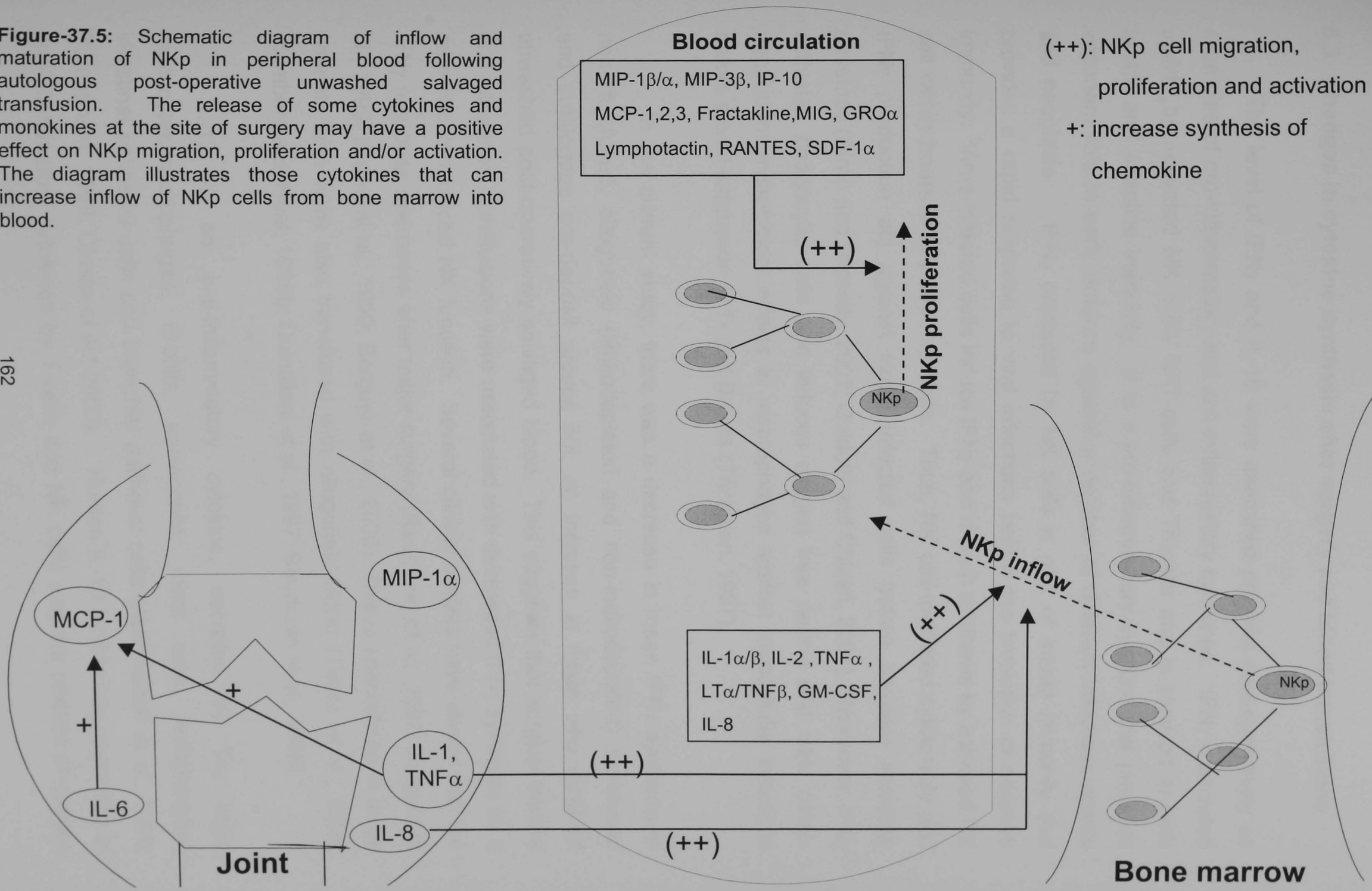
chemokine	subfamily	receptor	source	Target
GRO-α	CXC (α)	CXCR2	MQ/MO, endothelial cells	leukocyte, MQ/MO, NK cell, lymphocyte
IP-10	CXC (α)	CXCR3	MQ/MO, neutrophil, epithelial & endothelial cells	leukocyte, MQ/MO, NK cell, lymphocyte
IL-8	CXC (α)	CXCR1,2	MQ/MO, NK cell	leukocyte, MQ/MO, NK cell, lymphocyte
MIG	CXC (α)	CXCR3	MQ/MO, neutrophil, epithelial & endothelial cells	leukocyte, MQ/MO, NK cell, lymphocyte
SDF-1α	CXC (α)	CXCR4	BM stromal cells, endothelial cells	CD34+haematopoietic progenitor cell, leukocyte, MQ/MO, NK cell, lymphocyte
6Ckine	CC (β)	CCR7	MQ/MO, keratinocytes epithelial & endothelial cells	dendritic cell, NK cell, lymphocyte
I-309	CC (β)	CCR8	MQ/MO, lymphocytes, NK cell	leukocyte, MQ/MO, NK cell, lymphocyte
MCP-1,2,3	CC (β)	CCR2	MQ/MO, keratinocytes epithelial & endothelial cells	leukocyte, MQ/MO,NK cell, lymphocyte, platelet, mast cell, fibroblast
MIP-1α	CC (β)	CCR1,5	MQ/MO, keratinocytes, NK cell, endothelial cell, platelet	leukocyte, MQ/MO, NK cell, lymphocyte, fibroblast
MIP-1β	CC (β)	CCR1,5	MQ/MO, keratinocytes, NK cell, endothelial cell	leukocyte, MQ/MO, NK cell, lymphocyte, fibroblast
MIP-3β	CC (β)	CCR7	MQ/MO, keratinocytes, endothelial cells	NK cell, lymphocyte
RANTES	CC (β)	CCR1,5	T-,NK,NKT-cell, MQ/MO, endothelial cell, chondrocyte	leukocyte, MQ/MO, NK cell, lymphocyte
Lymphotactin (Ltn)	C (γ)	XCR1	NK cell, T-cell, mast cell	leukocyte NK cell, lymphocyte
Fractakline (neurotactin)	CX_3C (δ)	CX_3CR1	endothelial and epithelial cells, dendritic cell	T-cell and NK cell

3) Complement production. C5b is a component of the complement system produced during the formation of the C5b-9 membrane attack complex that causes destruction of target cells or micro-organisms. C5b is a chemotaxin and activator of neutrophils. It also mediates an inflammatory reaction at the site of injury by stimulating endothelial cells (Rawal and Pangburn, 2001).

Figure-37.5 summarises the possible effect of some cytokines on inflow of NKp into blood from bone marrow or the effect of some monokines in blood on NKp proliferation and activation following unwashed autologous post-operative salvaged blood.

Individual variation in NKpf was apparent both before and after operation presumably reflecting different clone sizes.

Figure-37.5: Schematic diagram of inflow and maturation of NKp in peripheral blood following autologous post-operative unwashed salvaged transfusion. The release of some cytokines and monokines at the site of surgery may have a positive effect on NKp migration, proliferation and/or activation. The diagram illustrates those cytokines that can increase inflow of NKp cells from bone marrow into blood.



(++): NKp cell migration, proliferation and activation

+: increase synthesis of chemokine

5.3. Changes in cytokine synthesis after surgery and blood transfusions

The level of IFNγ and IL-10 were examined pre and post-operatively as examples of pro-inflammatory and anti-inflammatory cytokines. IFNγ is secreted mainly by activated NK cells, NKT-cells and Th_1cells and is involved in both innate and adaptive immunity. It is a pro-inflammatory cytokine that plays an important role in early defence against a number of different infectious agents and neoplasia. IFNγ produced by NK cells is part of innate immunity that provides a rapid response to viral infections before the involution of adaptive immunity. Virus-infected cells induce IFNγ and IFNα/β synthesis by activated NK cell within hours of primary infections. Thus, NK cell-mediated cytotoxicity and IFNγ production act against virally infected cells before adaptive immunity develops (Biron and Brossay, 2001; Guidotti and Chisari, 2001). Moreover, IFNγ activates phagocytic cells and induces oxygen free radical and nitric oxide-dependent microbicidal effects in macrophages against intracellular infections before clonal expansion of T- and B-cells (Trinchieri, 1997).

In the current study, there was a decrease in mean IFNγ synthesis in non-transfused, allogeneic (leukodepleted and non-leukodepleted) transfused and autologous pre-deposit groups but, an increase in those who received unwashed post-operatively salvaged blood. This suggests that surgical trauma and allogeneic transfusions were associated with decreased IFNγ synthesis as a reflection of reduced NK function. Several clinical studies have shown that the IFNγ response decreases after major surgery (Redmond et al., 1992; Berguer et al., 1999; Brune et al., 1999; Berguer et al., 2000). This is specially so in those patients who were also transfused with allogeneic blood (Tietze et al., 1995; Blumberg and Heal, 1996b; Quintiliani et al., 1997; Baudouin et al., 1998).

IL-10 is an anti-inflammatory cytokine, secreted by Th_2 cells, monocytes/macrophages, B-cells, eosinophils, mast cells, keratinocytes, intestinal epithelial cells and bronchial epithelial cells (Autschbach et al., 1998; Romagnani, 1999; Okada et al., 2000). Human IL-10 inhibits the production of pro-inflammatory cytokines by T-cells and NK cells and the release of oxygen

free radicals and nitric oxide-dependent microbicidal activity by macrophages and neutrophils. It also inhibits antigen-specific activation, proliferation and the expression of CD54, CD80, and CD86 by T-cells (de_Waal_Malefyt et al., 1991b; Groux et al., 1998). However, other studies show that IL-10 increases NK cell cytotoxic function and cytokine release. These studies also show that NK cells express IL-10R and can produce IL-10 thereby inhibiting pro-inflammatory cytokines production within the vicinity of NK cells (Liu et al., 1994; Carson et al., 1995a; Mehrotra et al., 1998; Peritt et al., 1998).

In this study an attempt was made to correlate IL-10 synthesis with surgical trauma and different kinds of transfusion. The result obtained was that patients receiving unwashed post-operatively salvaged blood drained from the wound showed an increase in the IL-10 synthesis compared to the other groups. This was of interest firstly because, increased IL-I0 was assumed to decrease IFNγ synthesis. Secondly, because it begged the question of the source of IL-10 synthesis and whether this it was produced from NK cells or other cells in the culture.

There was a positive correlation between changes in NKpf and changes in IFNγ ($r=0.28$) and between NKpf changes and IL-10 changes ($r=0.16$) in all patient groups combined. Furthermore, there was a positive correlation between changes in IFNγ and changes in IL-10 ($r=0.13$) in all patient groups combined. Other studies support a correlation between the NK cell cytotoxic function and IFNγ synthesis in various patient groups (Quintiliani et al., 1997; Andersen et al., 1998; Bordin et al., 1999). In contrast, the IL-10 result was not supported by previous studies that mainly demonstrated a decrease in post-operative IL-10 production (Heiss et al., 1997b; Klava et al., 1997; Kirkley et al., 1998; Koller et al., 1998; Berguer et al., 1999; Berguer et al., 2000).

The present studies involved IL-2 and IL-15 activated PBMC cultures. Thus IL-10 could have been produced by Th_2 or NK cells. Liu and Carsons' studies showed that activated NK cells were able of producing IL-10 (Liu et al., 1994; Carson et al., 1995a). Joshi showed a significant decrease in NK cell

cytotoxic function when PBMC were cultured for 40 hours with plasma samples derived from trauma patients and this phenomenon was not reversed by adding neutralising anti-IL-10 antibody. However, removal of monocytes from the cultured cells significantly reversed the NK cell cytotoxic function (Joshi et al., 1998). This does not exclude the possibility that IL-2 activated Th_2 cells produced IL-10 in our studies. But it begs the question as to why more IL-10 was not produced in groups that showed post-operative suppression of NKp.

IL-10 acts by inhibiting prostaglandin E_2 (PGE_2) production and is one of the main regulators of the immune response. PGE_2 production increases after surgery and has a deleterious effect on NK cell function (Rees and Platts, 1983; Ertel et al., 1993; Wang et al., 1997; Kim et al., 1999; Rhind et al., 1999; Liu et al., 2000b; Deichman et al., 2001; Moore et al., 2001). Thus reduced IL-10 synthesis observed post-operatively in our studies may have contributed to increased PGE_2 production and decreased NK function. It also holds that the reverse is true following transfusion of autologous salvaged blood.

The IL-4 results are less reliable due to sample selection. Several previous studies reported similar results, whereby IL-4 synthesis increased after surgical trauma and allogeneic transfusions (Ayala et al., 1994; Coimbra et al., 1996; Mack et al., 1996; Levy et al., 1997; Baudouin et al., 1998; Zedler et al., 1999; Koga et al., 2000; Ochoa et al., 2001). IL-4 is produced mainly by Th_2 cells and is regulated by IFNγ produced by Th_1 cells (Romagnani et al., 1998). However observation in the current study showed a negative correlation between IL-4 and IFNγ synthesis.

5.4. Is CD56 adequate as a unique marker for functional NK cells?

NK cell cytotoxicity is an important indicator for evaluating baseline immune defence, monitoring progress during cancer immuno-therapy and treatment of AIDS.

Different methods are being used to assess NK cytotoxic function such as ^{51}Cr release assay, lactate dehydrogenase (LDH) release assay, Eu-release assay, Calcein cytotoxicity assay, flow cytometric assay of intracellular cytokine in NK cells, MTT assay (measuring the cell viability via mitochondrial activity in target cells) and ^{125}IUdR-release assay (measuring DNA fragmentation in target cell) (Morales and Ottenhof, 1983; Blomberg et al., 1986b; Blomberg et al., 1986a; Chang et al., 1993; Umemoto et al., 1997; Roden et al., 1999; Mendes et al., 2000).

In the flow cytometry assay the number of NK cells in the circulating blood is determined by double or triple staining with monoclonal antibody against CD3, CD16 and CD56. For precise enumeration of NK cells among PBMC cells the sample should be stained by a combination of anti-CD3 and anti-CD56 (double staining) or anti-CD16 (triple staining) (Mendes et al., 2000). Furthermore, it is possible to quantitate activated NK cells by flow cytometry by determining HLA-DR, CD25 (IL-2Rα), CD69 and CD71 (transferrin receptor) expression in the CD3-CD56+ cell population. However, it is not clear that the expression of these activation markers correlates with NK cell cytotoxic function (Whiteside and Herberman, 1994; Whiteside and Herberman, 1995b).

Attempts to relate immuno phenotype to NK cytotoxic function have been made by others (Roder et al., 1980; Whiteside and Herberman, 1989; Whiteside and Herberman, 1994). In humans CD16 exists in two isoforms: a 56-60 KDa trans-membrane isoform (FcγRIIIA) mainly expressed on NK cells, mast cells and monocytes/macrophages and a GPI-linked 48 KDa isoform (FcγRIIIB) expressed exclusively on neutrophils. CD16 may also be expressed on CD3+ T-cells in certain individuals. CD56 is expressed on some human haemopoietic cells

including NK cells and a sub-population of T lymphocytes termed NKT-cells. It is also expressed in adult neural tissues, muscle and embryonic tissues. A number of tumour cell types are positive for CD56 including some myeloid leukaemias, myelomas, neuroblastomas, Wilm's tumour and small cell carcinomas (Barclay et al., 1997; Carayol et al., 1998; Godfrey et al., 2000).

Observations in the present study suggested that rIL-2 alone preferentially activated CD3-CD16+ compared to CD3- CD56+ cells whereas rIL-15 activated a higher percentage of CD3-CD56+ population. This result suggests that although IL-2R and IL-15R can both be ligated by IL-2 and IL-15 by virtue of the shared β and γc chain, they reserve their highest specificity for their specific cytokines. Furthermore the result suggests these proliferation that are IL-2R+, IL-15R-; IL-2R+, IL-15R+ and IL-2R-, IL-15R+.

Lack of correlation between percent CD3-CD56+ cells and NKpf (r=0) was consistent with the notion that although most NK cells may express CD56+, not all cells expressing CD56+ exhibit NK function, and circulating CD56+ cells may vary in their state of cytotoxic activation (Whiteside and Herberman, 1995a). Also LDA assay measures the number of precursors of functionally active NK cells within a PBMC sample with very high sensitivity compared to flow cytometry assay, which enumerates the proportion of CD3-CD56+ cells within a PBMC sample with lower sensitivity.

These results support the previous observations that enumeration of NK cells by flow cytometry is unable to measure reliably NK cytotoxic function and that the CD3-CD56+ cell is an inadequate marker (Roder et al., 1980; Whiteside and Herberman, 1989; Whiteside and Herberman, 1994). Figure-38.5 is schematic summery of measuring NKpf by LDA and enumeration of percent CD3-CD56+ cells by flow cytometry.

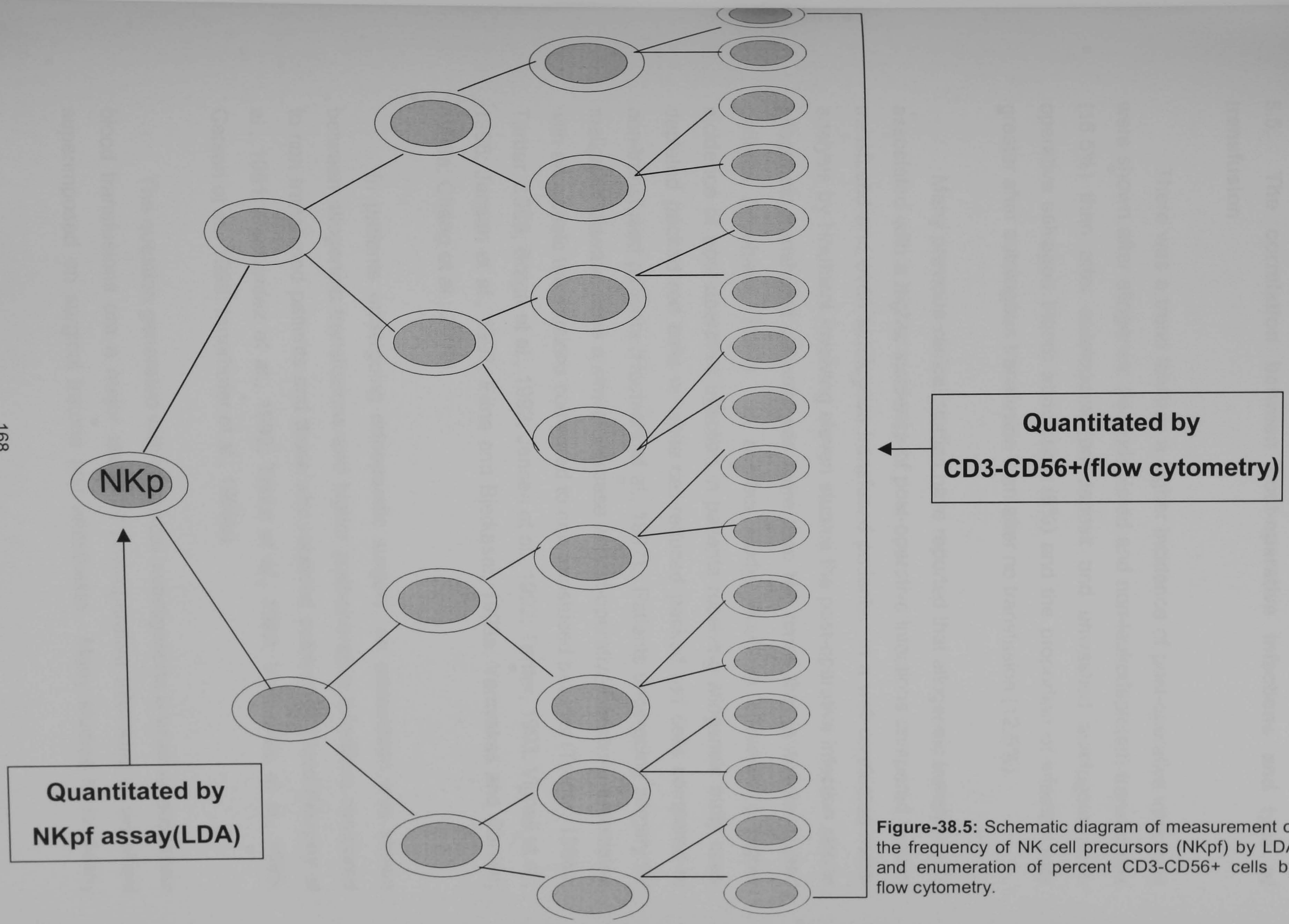


Figure-38.5: Schematic diagram of measurement of the frequency of NK cell precursors (NKpf) by LDA and enumeration of percent CD3-CD56+ cells by flow cytometry.

5.5. The correlation between post-operative infections and type of transfusion

There was a trend towards a higher incidence of post-operative infections were shown after allogeneic (leukodepleted and non-leukodepleted) transfusions (16.5%) than after autologous (pre-deposit and unwashed autologous post-operative salvaged blood) transfusion (8%) and the proportion of infection was greater after autologous transfusion than after no transfusion (12.5%).

Many previous clinical studies have reported that allogeneic transfusion is associated with a higher incidence of post-operative infections compared to non-transfused and even autologous transfused patients. In a well-conducted meta-analysis by Houbiers involving eleven studies the post-operative infection rate in allogeneic transfused patients was more than two times that in non-transfused patients (Houbiers, 1994). In another study, Houbiers observed a higher incidence of post-operative infection in patients receiving allogeneic buffy coat-depleted packed red cells or white cell-reduced packed red cells compared to non-transfused patients (Houbiers et al., 1997). Patients undergoing surgery for malignant disease have a similar increase in post-operative infections associated with allogeneic transfusions compared to non-transfused patients (Tartter, 1988a; Tartter, 1989; Braga et al., 1992; Jensen et al., 1992; Tartter, 1993; Vignali et al., 1996; Jensen et al., 1997; Edna and Bjerkeset, 1998a; Vamvakas and Carven, 1998a; Chang et al., 2000).

In patients undergoing orthopaedic surgery, an association was shown between allogeneic transfusions and higher post-operative infections compared to non transfused patients and those who received autologous blood (Murphy et al., 1991; Fernandez et al., 1992; Triulzi et al., 1992; Vamvakas et al., 1995; Carson et al., 1999; Innerhofer et al., 1999b).

The question generated with all clinical investigations is whether allogeneic blood transfusions are a major cause of post-operative infections or an effect superimposed on surgical trauma and anesthesia. Many studies have clearly

shown that major surgery is strongly associated with deleterious effects on the immune system leading to increased risk of post-operative infections (Rodrick et al., 1986a; Pollock et al., 1992; Griffith et al., 1995; Bruns et al., 1996; Hoffman et al., 1996; Kutza et al., 1997; Andersen et al., 1998; Berguer et al., 1999; Stanojevi‡_Baki‡ et al., 1999; Berguer et al., 2000). But, it is difficult to quantitative this result because of surgical and anaesthetic techniques, and even drugs are strikingly different from centre to centre.

Since there is strong evidence that NK cells play a key role in defence against intra-cellular micro-organisms, NKpf assays may reflect susceptibility to infection. However, clearly these assays take too long to be of routine prognostic value. Notwithstanding this NKpf changes may reflect major immunomodulatory effects of surgical trauma and/or blood transfusions. NKpf assays could also be used to explore the efficacy of a factor that clearly exists in wound drainage fluids that, if isolated, may reverse the immunosuppression induced by surgical trauma.

Other parameters of innate immunity including cellular components and/or soluble mediators such as PMN cells, monocytes/macrophages, complement and antibody may also have been affected by surgical trauma and/or allogeneic blood transfusions resulting in an increase in the risk of post-operative infections mainly by extra-cellular bacteria.

The results at the present study suggest that surgical trauma and allogeneic blood transfusions induce immunomodulation by impairment in NK cytotoxic function, decreased IFNγ synthesis and IL-10 synthesis and that these effects could be reversed by unwashed autologous post-operative salvaged blood.

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Appendix

Literature review of some studies

1. A retrospective study in colorectal malignancy patients showed that the proportion of tumour recurrence in those who received allogeneic blood was significantly more than patients non-transfused. In this study, the records of 177 transfused and 118 not transfused patients who underwent surgery for colorectal malignancy were examined. They observed that ninety-five percent (95%) of not transfused patients were recurrence free at the end of 1 year compared to eighty-six percent (86%) of those patients, who transfused before operation. This difference persisted for the 5 years of the study. In addition, transfused patients in any of the pathological stages had lower recurrence-free rates in every postoperative year compared to allogeneic transfused patients (Burrows et al., 1987).

2. Tartter is one of the most important pioneers to have increased awareness of the phenomena that allogeneic transfusion is associated with immunomodulation in the host immune system. He found significant depression of NK cell activity, higher risk for tumour recurrence, longer hospital stays, higher incidence of post-operative infection and higher hospital charges after allogeneic transfusion in colorectal cancer patients. In addition, he noticed that the mean number of units of allogeneic blood received by colorectal malignancy patients who developed infectious complications significantly exceeded the number for patients without infectious complications. Of the 343 patients who underwent surgery 134 patients received allogeneic transfusions. A total of 33 post-operative infections were identified in 33 of the 134 patients, giving an infection rate of 25 percent. By contrast, of the 209 patients who did not receive allogeneic transfusion 9 patients developed post-operative infection given infection rate of 4.3% (Tartter, 1988b; Tartter, 1988c; Tartter, 1988a; Tartter, 1992).

3. Blumberg in a series of papers has shown that allogeneic blood transfusion was not only associated with deleterious effects on host immune defences, but also with additional costs of up to $1500 per unit. Hospital charges were estimated in patients undergoing hip-replacement surgery. The main outcome was increased hospital costs per unit transfused. The mean of total hospital charges among donors of autologous blood was $4,800. Whereas, the mean of total hospital charges was $7,200 for recipients of allogeneic transfusions. Thus, each allogeneic transfusion was associated with additional cost of $1,480. Blumberg is one the pioneers who believe that allogeneic leukocytes in cellular blood products are the main reason why allogeneic transfusion induces immunomodulation. He has also suggested that leukodepletion of allogeneic blood reduced or prevented these deleterious effects. In addition, he speculated that the mechanism may involve altered cytokine regulation with a shift toward an anti-inflammatory response (Blumberg and Heal, 1987; Blumberg et al., 1996; Blumberg, 1997)

4. In two interesting studies on NK cells, in 1987, it was shown that blood donation was associated with a decrease circulating NK cell levels and function in the healthy volunteer. However, these effects was not associated with adverse clinical manifestations in the healthy blood donor (Ford et al., 1987; Lasek et al., 1987).

5. In 1990, was suggested that allogeneic blood was associated with later hospital discharge, a higher rate of post-operative infection and more antibiotic therapy compared to autologous blood transfusion. In this retrospective study, 190 patients who underwent hip replacement surgery were analysed. 34 patients were transfused with 2 or 3 units of autologous blood and 50 patients were transfused with 2 or 3 units of allogeneic blood. Other patients were excluded from the study for a number of different reasons. A total of 16 post-operative infections were identified in 16 of the 50 patients, giving an overall infection rate of 32 percent. By contrast, only one of the autologous transfused patients, (3%), developed post-operative infection. Thus, the proportion of post-operative infections in allogeneic transfused patients was 10 times more than the

autologous transfused groups. In addition, patients identified as being infected required significantly more antibiotic therapy (mean, 7.6 days) and lengthier hospital stays (mean, 15.5 days) than patients who remained free of infection (Murphy et al., 1991).

6. In a study that compared the effects of allogeneic and autologous blood transfusions on post-operative infection rates, allogeneic blood transfusion was associated with higher post-operative infection compared to autologous transfusion. Fifty patients received autologous blood and 50 allogeneic blood but underwent the same procedure. The proportion of post-operative infection, confirmed with positive cultures, was sixteen percent of the allogeneic transfused patients and 4 percent in the autologous patients (Mezrow et al., 1992).

7. In 500 patients who underwent surgery for pancreatic and gastrointestinal cancer the amount of blood transfused and the volume of per-operative blood lost were independent risk factors in the development of post-operatively infection (Braga et al., 1992).

8. In patients who underwent spinal fusion operations an increase in post-operative infection and longer hospital stay were found in patients receiving allogeneic transfusion compared to those who received autologous or no transfusion. They also noticed that surgical trauma, allogeneic blood transfusion and anaesthesia decreased the number of NK cells. Of 109 patients, 60 received autologous blood; 24 received at least one unit of allogeneic blood, and in 25 had no transfusions. A total of 22 post-operative infections were identified in 22 of 109 patients. These occurred while in hospital (8) and in after discharge (14) and gave an overall infection rate of 22.2 percent. Twenty-two patients developed bacterial infections. The post-operative infection rates were, 3.3 percent in autologous transfused patients, 20.8% in allogeneic transfused patients and 4 % in no transfused. In addition, multivariate logistic and linear regression analysis revealed that the number of allogeneic units transfused was the only significant predictor of in-hospital infection or days on antibiotics and length of stay (Triulzi et al., 1992).

9. In 1992, it was shown that allogeneic blood transfusion impaired NK cell function for up to 30 days after operation in patients who underwent colorectal surgery. Also, in this study the effect of leukodepletion and platelet depletion on post-operative infection rate was researched. Of 197 patients undergoing elective colorectal surgery, 48 received blood free from leukocytes and platelets and 56 received whole blood transfusion. The number of post-operative infections were, 13 in transfused patients with whole blood (23 %), one in patients transfused with filtered blood (2%) and two in the non transfused patients (2%) (Jensen et al., 1992). They also showed a decrease in lymphocyte proliferation and CD4+CD8+ ratios, and a higher proportion of post-operative infection in allogeneic blood transfused patients (Jensen et al., 1997).

10. In three different studies in patients who underwent colorectal surgery, Busch et al observed that allogeneic blood transfusion was associated with poor prognosis and a significantly higher risk of tumour recurrence compared to those who did not receive blood transfusion, either allogeneic or pre-deposit autologous. In a randomised study on 475 patients who underwent colorectal cancer the risk of recurrence was investigated. 236 patients out of a total of 475 patients received allogeneic transfusions and 239 patients received autologous pre-deposit transfusion. Patients in the autologous-transfusion group were required to donate two units of blood before their surgery. The cancer-specific survival rates at four years were 67 percent in the allogeneic transfused patients and 62 percent the autologous pre-deposit transfused group. Thus, no significant difference in prognosis was observed. By contrast, the risk of recurrence was significantly increased in the allogeneic or autologous pre-deposit groups, as compared with non-transfused patients (Busch et al., 1993b; Busch et al., 1994b; Busch et al., 1994a). In the same study, the effects of allogeneic and autologous pre-deposit blood transfusions on patients who underwent colorectal cancer were investigated. The post-operative infection rate with autologous was significantly less than allogeneic blood transfused patients. In this study of 120 patients, 58 patients donated their blood before operation. The number of post-operative infections was 7 (12%) in transfused autologous pre-deposit patients and 17(27%) in the allogeneic transfused patients. Thus, the proportion of post-

operative infection in allogeneic transfused group was 2.25 times more than autologous pre-deposit transfused group, despite the similarity between the groups in risk factors known to affect post-operative infections (Heiss et al., 1993).

11. A meta-analysis confirmed these observations and provided the foundation for a prospective randomised control trial. This showed that allogeneic transfusion was associated with increased mortality due to post-operative infection but the increase was not due to recurrence of the colorectal cancer. In this study, the data from 871 patients with colorectal cancer was collected from 16 hospitals. Patients, who received allogeneic transfusions of any, sort (leukocyte-depleted red cells or packed cells without buffy coat) had a lower three-year survival and a higher infection rate than non-transfused patients. This confirmed that allogeneic transfusion, leukocyte-depleted red cells or packed cells without buffy coat, were associated with poor patient survival. By contrast, colorectal cancer recurrence rates, were not influenced by blood transfusion (Houbiers et al., 1994a).

12. In 27 patients who underwent hip replacement surgery the effects of allogeneic and autologous buffy coat-depleted red cells on cytokine levels in serum were investigated. The result showed a decrease in IL-2 and IFNγ serum levels, and an increase in IL-6 levels post-operatively in both groups (Tietze et al., 1995).

13. In a big review study by Landers, he found strong evidence that allogeneic transfusion was associated with an increase in cancer recurrence, post-operative infections and longer hospital stays (Landers et al., 1996).

14. In a prospective study on 267 patients who underwent colorectal cancer 47 of 142 patients receiving allogeneic transfusion developed post-operative infection. Multivariate analysis identified allogeneic transfusion as the only variable related to the occurrence of postoperative infections (Vignali et al., 1996).

15. In a study, of NK and LAK cell (lymphokine-activated killer) activity, allogeneic transfusion was associated with post-operative decrease in NK cells and LAK activity. By contrast, autologous transfusion was associated with increased NK cell activity (Heiss et al., 1997a).

16. A significant reduction of NK cell cytotoxic function against K562 target was observed in allogeneic transfused patients was observed compared to patients who did not receive transfusions (Mathiesen et al., 1994)

17. Both leukodepleted and non-leukodepleted blood transfusions impaired NK cell function post-operatively. Of 129 patients who underwent surgery for colorectal malignancy, 42 received transfusions. Of these 42, 21 received leukocyte-depleted products and 21 non-leukocyte-depleted products. A significant reduction in %CD4+ lymphocytes at 6 months post-operatively was observed in the latter group. In contrast, no significant changes in %CD4+ lymphocytes were observed in leukocyte-depleted transfused patients. There was a marginal decrease in HLA-DR+ lymphocytes in the non-leukocyte-depleted transfused patients without a history of previous transfusion (Mathiesen et al., 1998).

18. In a literature review, it was speculated that allogeneic red cell transfusion was a risk factor for impairment of phagocyte cells in bacteria clearance. Post-operative infection in 697 patients with colorectal cancer who received buffy coat-depleted packed red cells and white cell-reduced (filtered) packed red cells, were compared with non transfused patients. The proportion of post-operative infections with both types of red cells was greater than the non-transfused group (39 vs. 24%, $p < 0.01$). In addition, multivariate analyses identified that red cell transfusion and tumour location were the only significant independent risk factors for postoperative bacterial infection (Houbiers et al., 1997).

19. In studies of 446 patients who underwent resection of colorectal adenocarcinoma, allogeneic blood transfusion was an independent risk factor for

the development of post-operative hospital infections (Edna and Bjerkeset, 1998a). They also studied 336 patients who underwent colorectal surgery for adenocarcinoma and showed that there was a significant correlation between per-operative transfusion and death (Edna and Bjerkeset, 1998b).

20. A study of 487 patients who underwent colorectal cancer surgery speculated that later discharge from hospital and higher hospital charges were associated with a higher post-operative infection rate due to allogeneic transfusion. The mean length of stay in hospital was, 16.7 days in transfused patients and 10.3 days in the non- transfused patients. The mean hospital charge was a $28101 in transfused patient and $15978 in non-transfused patients. The length of stay in hospital was increased by a average of 1.3% per unit of blood transfused ($p<0.05$) and hospital charges increased by 2.0% per unit of blood transfused ($p<0.05$) (Vamvakas and Carven, 1998a).

21. Innerhofer et al indicated that transfusion of buffy-coat depleted blood was associated with increased post-operative infections in orthopaedic patients. 385 patients undergoing elective surgery were transfused with either allogeneic buffy coat-depleted or autologous transfusions. The proportion of post-operative infections was, 4.6 % in the non-transfused or autologous groups and 11.9 % in the allogeneic transfused group (Innerhofer et al., 1999b). They also showed impairment of cell mediated immunity after allogeneic transfusion in recipients with changing T-cell proliferation and T-cell balance (helper-suppressor ratio). T-cell proliferation, PHA stimulation and mixed lymphocyte culture, were all significantly decreased in transfused allogeneic leukocyte-reduced compared to the autologous transfused group. This reduction persisted up to 10 days after transfusion. A trend toward decreased T-cell proliferation was observed in allogeneic buffy coat-depleted transfused patients. The number of CD4+ T-cells was also significantly lower in both kinds of allogeneic transfusions compared to autologous transfusion. In addition, a decrease in the numbers of CD3+ T-cells and an increased CD4:CD8 ratio was observed in allogeneic transfused patients (Innerhofer et al., 1999a).

22. Other studies supported the idea that allogeneic transfusion alters host cellular immunity by decreasing the number of lymphocytes and NK cells. In Bordin's study, the effect of leukodepletion and non-leukodepletion on allogeneic transfusions on host cell-mediated immunity, was evaluated. Using PHA stimulation of 47 patients undergoing hip replacement surgery, 17 received allogeneic transfusion and 16 were non-transfused. The post-operative lymphocyte and NK cell numbers were significantly decreased in those transfused with 3 or more units of blood, compared with non transfused patients. Post-operatively, IL-2 and IFNγ synthesis following PHA stimulation was decreased in transfused patients. In another study, 51 women with stage II breast cancer who underwent surgery were studied. The CD3+, CD4+, CD8+, and CD20+ cells were analysed by flow cytometry before surgery and three weeks after operation. No significant differences between pre- and post-operative lymphocyte subset levels were seen in non-transfused patients but there was a significant increment in the CD8+ cell count, a decreasing CD4+ cell count and decreased CD3+ cell count levels in the transfused group ($p< 0.05$) compared with the non-transfused patients (Bordin et al., 1999; EroŸlu et al., 1999).

23. A retrospective cohort study in patients who underwent hip replacement showed a correlation between allogeneic transfusion and a higher incidence of post-operative bacterial infection. The records of 9598 patients undergoing surgical repair for hip fracture were evaluated. 58% of patients received at least one allogeneic blood transfusion. There was a serious post-operative bacterial infection in 437 patients (4.6%) of whom 28.8% died during their hospital stay. The most common site of infection was urinary tract, (12.1%). Pneumonia occurred in 3.8% of patients. The hospital charges in those who developed post-operative infections was $14,000 more than those with no infection. In total, transfusion was associated with a 35% higher risk of post-operative bacterial infection and a 52% greater risk of pneumonia (Carson et al., 1999).

24. In a prospective study, 1,349 patients undergoing colorectal surgery in 11 centres across Canada were analysed. Of these 282 were transfused with a total of 832 allogeneic blood units. The incidence of post-operative infection was significantly higher after allogeneic transfusion. The proportion of post-operative infections was, 25.9% after allogeneic transfusion and 14.2% after no transfusion. The most common sites of post-operative infections were the wound area and intra-abdominal sepsis. Multiple regression analysis identified allogeneic transfusion as a significant independent predictor for post-operative bacterial infection (Chang et al., 2000).

25. In a recent study of 442 patients who underwent colorectal surgery and received either allogeneic leukodepleted or non-leukodepleted transfusion. The proportion of post-operative infections were 45% in leukodepleted group, 38% in non-leukodepleted group and 22% in non-transfused group (Titlestad et al., 2001).

Surgery

26. In clinical studies of 18 patients with severe traumatic injury a significant decrease in IL-2 production up to 50 days after injury was observed (Rodrick et al., 1986b).

27. In 85 patients who underwent surgical resection for solid tumours a significant decrease in NK cell cytotoxic function was observed (Pollock et al., 1992).

28. In 34 patients who underwent laparoscopic cholecystectomy less depression of cell-mediated immunity was observed than those with open cholecystectomy (Griffith et al., 1995).

29. In clinical studies a significant decrease in NK cell cytotoxic function occurred in two groups of patients who underwent esophageal carcinoma and non-malignant thoracic and abdominal surgery (Bruns et al., 1996).

30. In a study of tumour recurrence and survival in patients undergoing laparoscopic and open colorectal cancer surgery for primary colorectal cancer, no adverse patterns of recurrence or survival was observed in the laparoscopic method compared to another one (Hoffman et al., 1996).

31. In rat and pig DTH response after laparoscopy and open colon resection was investigated. There was a higher and better preserved DTH response after laparoscopic resection than after open colon resection (Bessler et al., 1994; Trokel et al., 1994).

32. In mice the effects of laparoscopic and open laparotomy bowel resection on tumour establishment and growth were investigated by Allendorf et al. Tumours were more easily established and grew more aggressively after open laparotomy than laparoscopy (Allendorf et al., 1995a; Allendorf et al., 1995b; Allendorf et al., 1996; Allendorf et al., 1998).

33. A study on NK cell function and general anesthesia found that trauma, due to surgery and general anesthesia, has deleterious effects on NK cell activity. In this study, the peripheral blood mononuclear cells (PBMC), one hour pre-operatively and 20-24 hour post-operatively, were assayed for basal and IFN-alpha-stimulated NK cytotoxic function, in patients under isoflurane/N_2O anesthesia. The basal NK cytotoxic function against K562 declined significantly after general anesthesia and surgery. However, the post-operative decrease in NK function was reversed after IFNα treatment (Kutza et al., 1997).

34. Another study on NK cells in patients following diagnosis of breast cancer and surgery showed that trauma, due to operative stress was associated with decreasing NK and T-cell activity. NK cell and T-cell activity were assayed in 116 patients treated surgically for invasive breast cancer. A lower NK cell cytotoxic activity and lower NK cell response to IFNγ was observed. A lower proliferative response of peripheral blood lymphocytes to plant lectins and to a monoclonal antibody directed against the T-cell receptor, was also found (Andersen et al., 1998). Furthermore, in another study in breast carcinoma patients, a post-operative decrease in lymphoproliferation and NK cell function was shown (Stanojevi‡_Baki‡ et al., 1999).

35. In a study by Berguer and his colleagues the cytokine synthesis after open and laparoscopic surgery, were compared. Intracellular T-cell IFNγ, IL-4, and IL-10, along with serum IL-6, were immediately measured before and one day after laparoscopic cholesystectomy. There was a slight increase of intracellular levels of IFNγ in stimulated T-cell subsets after surgery. However, there were no post-operative changes in stimulated IL-4 or IL-10 levels. Post-operative serum IL-6 levels were significantly elevated compared to pre-operative values. In total, major open surgery influences T-cell intracellular IFNγ, IL-4, and IL-10 more than laparoscopic cholesystectomy (Berguer et al., 2000).

36. Surgical trauma is associated with decreased Th_1 cytokine production. The in-vitro cytokine synthesis was measured immediately before and for 2 days after aortic surgery or carotid endarterectomy. The levels of IL-2

and IFNγ were significantly depressed during the post-operative period in all T-cell subsets. There was no post-operative increase in IL-4 or IL-10 synthesis (Berguer et al., 1998; Berguer et al., 1999).

37. In a clinical study, significant suppression in NK cell function and IL-2 production were observed after severe burning (Rodrick et al., 1986a).

38. In mice it was shown that IL-2 production in splenocytes stimulated by concanavalin-A was suppressed up to 21 days following burns. Increased IL-2 production was observed after adding Indomethacin to the cell culture (Wood et al., 1987).

39. In a clinical study by Blazer the effect of burn injury on NK cell function was investigated. The more severely burned patients had significantly depressed NK cytotoxic function persisting for as long as 40 days following burning injury. By contrast patients with less burns showed a decreased NK cell cytotoxic function persisting for 10 days following burning injury (Blazar et al., 1986).

40. Another clinical study showed that the alteration in neutrophil function after burn injury may be caused by LPS soon after injury and by C5a later after injury (Moore et al., 1986; Davis et al., 1987).

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Quantitation of natural killer cell precursors in man

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Abstract

A technique was developed to measure the frequency of natural killer cell precursors (NKpf) in human peripheral blood mononuclear cell (PBMC) samples. Functional maturity of NK cells was reflected in their ability to lyse target cells from the K562 cell line. During the development of the technique, venous blood was taken from one healthy adult and assayed at different times to avoid individual variation. The technique was based on the principle of limiting dilution analysis. The NKpf assay was set up with a range of cell dilutions from 40,000 to 625 per 100 μl/well in 96-well culture plates. At the end of the culture period, the K562 cell line labelled with europium (Eu-K562) was added and the Eu-release was measured in culture supernatants using time-resolved fluorometry. The NKpf value differed between individuals and was influenced by the length of time in culture, being maximal at day 5. Maturation of NKp required the continuous presence of recombinant interleukin 2 (rIL-2), or rIL-15, both being equally effective. In the absence of cytokines, the functional NK cells declined rapidly beyond 24 h in culture. Irradiated allogeneic cells appeared to substitute in part for cytokines, but the numbers of allo-activated NKpf were lower than those observed when allo-activated NKpf were cultured with rIL-2. This suggested selective activation by the allogeneic stimulus of subsets of NKp or rIL-2-rescue of NKp subsets destined for apoptotic cell death. Alternatively, the increased frequency could have been attributable to activation of precursors of natural killer-T cells (NK-Tp). This assay is suitable for estimating the total number of precursors of functional NK cells in the blood of patients. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Natural killer cell precursor frequency; rIL-2; rIL-15; Europium release; Limiting dilution analysis

1. Introduction

Maturation pathways of human natural killer (NK) cells are complex and poorly understood. T cells and NK cells are both derived from haemopoietic stem cells, but NK cells develop primarily but not exclusively along non-thymic pathways (Sivakumar et al., 1998; Punzel et al., 1999; Raulet, 1999). Although qualitative assays have been widely applied, few quantitative assays have been developed to measure the precursors of functional human NK cells. Long-term bone marrow cultures showed that CD3 – CD56+ CD7+ NK cells developed from CD34+ HLA DR – bone marrow cells in the presence of rIL-15 and stem

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cell factor. They were cytotoxic against target cells from the K562 cell line, a functional hallmark of mature NK cells that we have adopted in this assay. These cells express the characteristic CD3 – CD56+ phenotype of NK cells, but not the T cell (CD3+ CD5+ TcR), monocyte (CD14+ CD15+) or (CD19+) B cell phenotypes. (Carayol et al., 1998; Yu et al., 1998; Miller et al., 1999; Sato et al., 1999). Others have demonstrated that the early human haemopoietic stem cell phenotype (CD34+ CD3 – CD8 – CD56 –) yielded NK cell clones in the presence of rIL-15, suggesting an important role for this cytokine in the development and maturation (Mrozek et al., 1996; Yu et al., 1998; Liu et al., 2000). The distinction between NK cells and NK-T cells that have some of the hallmarks of thymus processed cells is unclear, and we have not attempted to distinguish between these subsets in this study (Sato et al., 1999).

Functional assay systems other than lysis of the K562 target cell line have indicated that NK cells play a vital role in defence against bacterial infection, virus-infected cells and malignant cells. Patients with reduced or no NK activity have a high frequency of infections and cancer (Herberman, 1981; Pross and Lotzova, 1993; Kos, 1998; Gosselin et al., 1999). Normal mice infected with lymphocytic choriomeningitis virus show peak NK cell activity on day 3 following infection, whereas a peak of CD8+ T cell activity is seen on day 7. As they differentiate, human NK cells secrete inflammatory cytokines such as interferon gamma (IFN-γ), tumour necrosis factor (TNF-α), IL-3, IL-8 and granulocyte/macrophage colony stimulation factor (GM-CSF) (Carson et al., 1995; Kos, 1998; Nguyen et al., 1998; Biron et al., 1999). In certain culture conditions, NK cells also produce the anti-inflammatory cytokines, IL-5, IL-10 and IL-13 (Warren et al., 1995; Mehrotra et al., 1998; Peritt et al., 1998; Hoshino et al., 1999).

Activated T cells producing IL-2 are able to drive NK cell activation and proliferation through the α, β and γ sub-units of the IL-2 receptor (IL-2R) expressed on the surface of NK cells (Voss et al., 1992; Carson et al., 1994; Eicher and Waldmann, 1998; Waldmann et al., 1998). The IL-15R has a unique α chain but shares the β and γ chains of the IL-2R, thus accounting for the shared signalling activity observed by these two cytokines (Carson et al., 1997; Waldmann and Tagaya, 1999). In the presence of excess IL-2, human NK cells become lymphokine-activated killer (LAK) cells that lyse fresh tumour target cells through a mechanism independent of MHC restriction. In vivo-activated NK cells are able to migrate through solid tissues, localise to the sites of metastases, and kill tumour cells (Pavletic et al., 1993; Okada et al., 1996; Tomita et al., 1998).

Recent reports suggest that IL-15 shares many of biological properties of IL-2 despite the absence of sequence homology. Activated monocyte/macrophage, bone marrow stromal cells and dendritic cells produce IL-15 (Carson et al., 1994; Seidel et al., 1998; Waldmann et al., 1998). Several reports have described how mice lacking the IL-2 gene develop NK cells, whereas mice and humans lacking the γ sub-unit of the IL-2R have no NK cells. IL-15 uses its own IL-15Rα chain when binding with high affinity to IL-15R thereby signalling the NK cells to produce IFN-γ, GM-CSF and TNF-α (Noguchi et al., 1993; DiSanto et al., 1995; Carson et al., 1997). In vitro studies have demonstrated that IL-2 and IL-15 can activate human NK precursors cells and enhance the cytotoxicity of neonatal NK cells against K562 targets and virus-infected cells (Gaddy and Broxmeyer, 1997; Loubeau et al., 1997; Gosselin et al., 1999). In vivo IL-2 is only transiently produced by antigen-activated T cells, which in turn express high affinity IL-2R. IL-15 is produced by activated monocyte/macrophages, bone marrow stromal cells, dendritic cells and keratinocytes (Blauvelt et al., 1996; Jonuleit et al., 1997; Waldmann and Tagaya, 1999).

In this study, we have attempted to develop and standardise a non-radioactive europium (Eu)-release limiting dilution analysis (LDA) initially described in outline elsewhere (Blomberg et al., 1986a,b; Nagao et al., 1996) and to quantitate the frequency of natural killer cell precursors (NKpf) present in human peripheral blood.

2. Materials and methods

2.1. Subjects studied

The experiments described in this study were fully approved by the local hospital research ethics committee, and informed consent was obtained from all volunteers. Experiments designed to investigate the

factors influencing NKpf estimates were performed with blood from one healthy male adult. Other experiments to investigate individual variation were performed on samples obtained from 15 Caucasian patients aged between 45 and 83 who were awaiting surgery for osteoarthritis. Patients were excluded from the study if they had pre-existing infection, previous blood transfusion, malignancy, autoimmune disorders and diabetes.

2.2. Collection and isolation of blood

Twenty milliliters of whole blood was collected in a sterile tube containing preservative-free sodium heparin (25 units/ml of blood). Peripheral blood mononuclear cells (PBMC) were isolated within 12 h of collection by density gradient centrifugation (Lymphoprep of specific gravity 1077, Nycomed). After isolation of PBMC, they were cryopreserved in liquid nitrogen until required. To prevent ice crystal damage, PBMC were frozen in a 1:1 mixture of complete culture medium (CCM) consisting of RPMI 1640 (Sigma) supplemented with 50% heat-inactivated fetal calf serum (Sigma) and 3 mM L-glutamine (Sigma), and 20% dimethyl sulfoxide (DMSO) in RPMI 1640 (Sigma). Penicillin (100 units/ml) plus streptomycin (100 μg/ml) (Sigma) were also added to the medium to prevent bacterial contamination.

The cells were cooled in the nitrogen vapour at approximately −1 °C/min, using a 'plug' inserted into the neck of the Dewer flask. After 2 h, cryovials (Nunc, Denmark) were transferred to racks stored in the vapour phase of a large liquid nitrogen freezer. For allo-stimulation experiments, a pool of PBMC taken from four HLA-disparate, healthy apheresis donors were irradiated (30 Gy) and added to the culture as described below.

The HLA negative NK sensitive human erythroleukaemic K562 cell line (ECACC, UK) was used as target for quantification of functional NK cells. The cell line was grown in stationary cell suspension cultures consisting of RPMI 1640 supplemented with 2% heat-inactivated AB serum and 3 mM L-glutamine (Sigma) in vented tissue culture flasks (25 cm^2, Falcon). Antibiotics were not added as this reduced assay sensitivity. To ensure optimal growth, culture media were replaced on the second day and harvested on days 4–5. The cell line was regularly screened for mycoplasma using a Mycloplasma Test Medium Kit (Sigma).

2.3. NKpf assay

The complete culture medium (CCM) used throughout consisted of RPMI 1640 (Sigma) supplemented with 10% heat-inactivated human AB serum from normal healthy male donors (Sigma) and 3 mM L-glutamine (Sigma). Antibiotics were not added to CCM as this reduced assay sensitivity. rIL-2 (Eurocetus, Amsterdam) diluted in CCM was added to cultures in a final concentration of 25–50 Cetus unit ml. The rIL-15 (R&D, UK) was similarly diluted to a final concentration of 10 or 20 ng/ml depending on the experimental conditions. Both rIL-2 and rIL-15 were added on day 0 when setting up the assay and during feeding on day 5.

PBMC were rapidly thawed in a 37 °C water bath and the viability of cells was checked by staining with trypan blue and counting them by phase-contrast microscopy. Although the yield of viable cells after thawing was not calculated in all instances, the general experience was that approximately 90% of intact thawed PBMC identifiable by phase-contrast microscopy were judged to be viable on the basis that they excluded trypan blue. Viable PBMC were titrated in seven two-fold dilutions using 24 replicates per dilution in CCM starting with a concentration of 4×10^4 and finishing with 0.0625×10^4 PBMC per 100 μl/well in Falcon 3077 96-well round-bottomed culture plates. The baseline control consisted of 100 μl of CCM alone without PBMC. Where specified, an irradiated allogeneic pool of PBMC was added in a single concentration of 50,000 cells per 100 μl/well to responders on day 0 and incubated. All cultures were assayed following 18 h and 5, 7 or 10 days incubation at 37 °C in 5% CO_2 in a humidified atmosphere. The 7- and 10-day assays were refreshed with CCM on day 5.

2.4. Quantitation of cytotoxicity

The K562 cell line was transferred from culture flasks into a 50-ml polypropylene tube (Falcon) and centrifuged at $400 \times g$ for 10 min in the cold (4 °C) then washed twice with phosphate buffer saline (PBS). Cell counts were performed after the first wash to cal-

culate the amount of europium (Eu) to be added. One milliliter of labelling buffer was added for each 5×10^6 K562 cells. Labelling buffer consisted of 1 ml HEPES buffer (Sigma) plus 40 μl of a solution of 100 μM diethylene triamino pentacetate (DTPA, Sigma), plus 20 μl of 20 μM solution of europium chloride ($EuCl_3$, Fluka). Then, 50 μl of a solution of 500 μM dextran sulphate (Fluka) was added. The suspension was incubated for 15 min at 4 °C with gentle and thorough mixing. Then, 30 μl of 100 mM solution of $CaCl_2$ was added to stop the labelling reaction, and the suspension was incubated for a further 10 min at 4 °C. The K562 cell suspension was then washed four times with wash buffer (RPMI 1640 plus 3 mM L-glutamine plus 79 μl/50 ml of 1 M $CaCl_2$) and centrifuged at 300 × *g* for 6 min at 4 °C. Thereafter, cells were washed twice with CCM centrifuging at 400 × *g* for 6 min at 4 °C. A cell count was performed to calculate the approximate number of Eu-DTPA labelled cells to be added, and Eu-DTPA labelled cells (Eu-K562) were re-suspended in CCM at 10^5 cells/ml.

The last stage of the NKpf assay was performed by adding 10,000 Eu-K562 in 100 μl to each well of a 96-well culture plate. Plates were briefly centrifuged at 200 × *g* at room temperature for 1 min to accelerate the reaction and incubated for 3 h at 37 °C in 5% CO_2 in a humidified atmosphere. Plates were centrifuged for 5 min at 600 × *g* at room temperature and 20-μl aliquots of supernatant from each well were then transferred to a new 'reader plate' (Nunc) pre-filled with 200 μl of enhancement solution (Delfia, Finland). Finally, europium release was evaluated in a time-resolved fluorometer (Delfia) and the results were expressed in counts per second (cps) as described in detail elsewhere (Blomberg et al., 1986a,b; Nagao et al., 1996). Spontaneous release was determined by incubating target cells with CCM only and maximum release was obtained by lysing the target cells with 2% Triton X-100.

The mean and standard deviations between replicate wells of the baseline culture were calculated and any well that exceeded the mean cps plus three times the standard deviation was scored positive. The percentage of negative wells at each responder cell dilution was calculated and plotted against the responder cell concentration per well. The calculation of the mean NKpf values plus the standard error (SE) and the 95% confidence intervals (95% CI) was facilitated by a computer program (Strijbosch et al., 1987, 1988). Estimates were expressed as mean NKpf per 10^6 PBMC plus/minus the SE and plotted on a $\log_{10}$ scale as mean NKpf with 95% confidence intervals (CI). The significance of the difference in mean NKpf for each group was analysed with the two-tailed Student's *t*-test.

3. Results

3.1. Effect of culture duration and timing of rIL-2 addition on NKpf estimates

All initial experiments used to define the factors influencing NKpf were performed with PBMC from a single individual, AG. The lower limit of detection in this assay was 1 NKp per 10^6 PBMC. The maximum NKpf detected in AG blood after 7 days of culture was 2450 NKp per 10^6 PBMC (0.25%), obtained from experiments with rIL-15 (see Fig. 3 below). Initially, we showed that in the absence of rIL-2, NKpf decreased rapidly from a mean ± SD of 63 ± 6 to 3 ± 1 per 10^6 PBMC between days 1 and 10 (Fig. 1a). By contrast, when rIL-2 was added, NKpf increased rapidly from 105 ± 4 to 1272 ± 141 between days 1 and 10 (Fig. 1b). The increment between days 1 and 5 implied rapid maturation of non-functional precursor NKp. When no extra IL-2 was added on day 5, there was no significant increment in NKpf between days 5 (776 ± 151), 7 (816 ± 174) and 10 (821 ± 198) per 10^6 PBMC (Fig. 1c). However, after adding extra rIL-2 on day 5, a small but significant increment in NKp was observed between days 5 (782 ± 160) and 10 (1172 ± 131), implying that cultures contained NKp at different stages of maturation ($p < 0.05$ between days 5 and 7 and days 5 and 10) (Fig. 1d) and that the late maturing NKp fraction became IL-2-sensitive only during the later period of culture.

In most cases, NKpf reached a plateau between days 7 and 10, suggesting that all-available rIL-2-sensitive precursors had been activated. These results confirmed that maturation and proliferation of NKp and maintenance in culture was dependent on the continued presence of rIL-2, and that the full potential NK function was reflected in assays cultured to maturity over 7 days. Notwithstanding this observa-

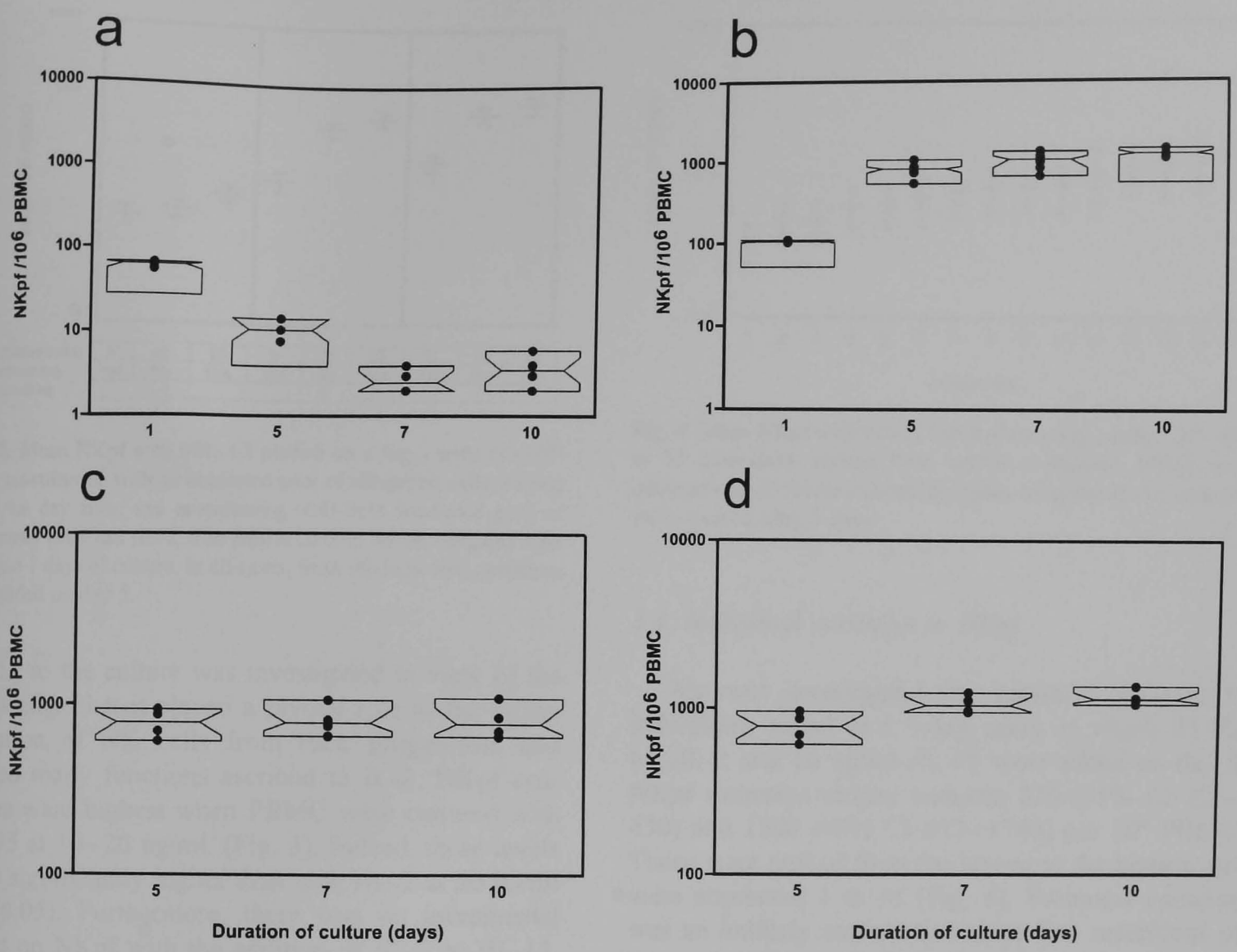

Fig. 1. Mean NKpf with 95% confidence intervals (95% CI) plotted on a $\log_{10}$ scale ($1-10^4$, figures a and b; 10^2-10^4, figures c and d) at different times of culture: (a) no added cytokines; (b) rIL-2 added on days 1 and 5 together with fresh medium; (c) rIL-2 added on day 0 but only fresh medium on day 5; (d) rIL-2 added on day 0, and day 5 in fresh medium. The values shown are results of three to seven experiments.

tion, all ensuing assays were terminated at day 5 unless otherwise stated, the rationale being technical simplicity and greater sensitivity during the clonal expansion phase.

3.2. Allo-activated NKp

The effect of activating NKp with a pool of irradiated allogeneic cells on day 0 was investigated. When allogeneic cells were added, an estimate of 190 ± 82 per 10^6 PBMC was obtained on day 7. However, when rIL-2 was added, this NKpf value rose to 1919 ± 307 per 10^6 PBMC (Fig. 2), suggesting that rIL-2 rescued certain NK progeny destined for apoptotic cell death. Alternatively, allo-activation may have stimulated a separate subset of NKp termed NK-T cell precursors (NK-Tp) having the hallmarks of both thymus processed T and non-thymically processed NK cells (Godfrey et al., 2000). To explore this concept, allo-activation was estimated on days 1, 5 and 7. A similar increment ($p < 0.001$ between rIL-2 alone and rIL-2 plus allo-activation) was observed on days 1, 5 and 7, supporting the view that allo-activation was associated with an additional subset of functional NK-Tp cells. These observations were confirmed in a further five experiments.

3.3. Effect of rIL-15 on NKpf

Recombinant interleukin 15 (rIL-15) was a candidate cytokine that may have accounted for some of the allo-activation effect. Therefore, the effect of adding

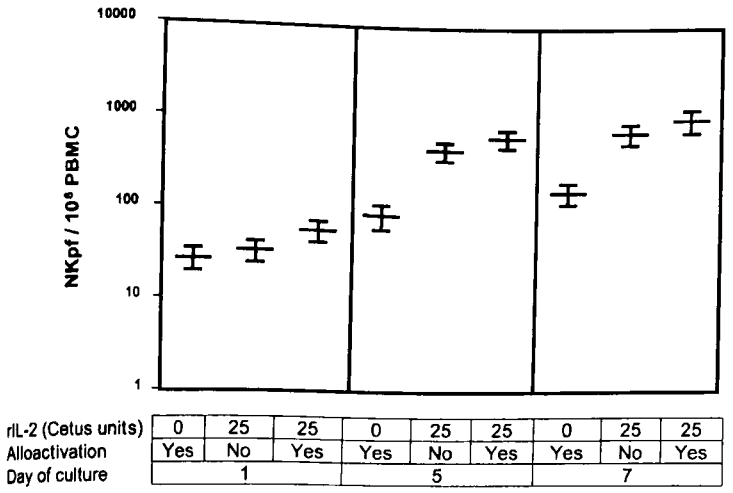

Fig. 2. Mean NKpf with 95% CI plotted on a $\log_{10}$ scale ($1-10^4$) after: co-culturing with an irradiated pool of allogeneic cells; adding rIL-2 on day zero; and co-culturing with both irradiated pool of allogeneic cells and rIL-2. The panels contain results obtained after 1, 5 and 7 days of culture. In all cases, fresh medium with cytokines was added on day 5.

rIL-15 to the culture was investigated in view of the knowledge that it played a pivotal role in the differentiation of NK cells from their progenitors and shared many functions ascribed to IL-2. NKpf estimates were highest when PBMC were cultured with rIL-15 at 10–20 ng/ml. (Fig. 3). Indeed, these levels were significantly higher than with rIL-2 at 50 IU/ml ($p<0.05$). Furthermore, there was no incremental effect on NKpf with the addition of rIL-2 to rIL-15. The rIL-15 alone appeared to be significantly ($p<0.05$) more effective than rIL-2 in driving the differentiation of NKp to maturity. This was confirmed in a further four repeat experiments.

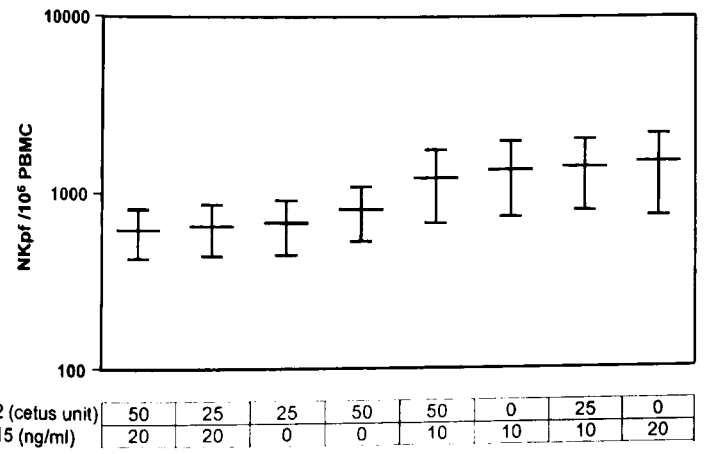

Fig. 3. Mean NKpf with 95% CI plotted on a $\log_{10}$ scale (10^2-10^4) after adding different concentrations and combinations of rIL-2 and rIL15 on days 0 and 5.

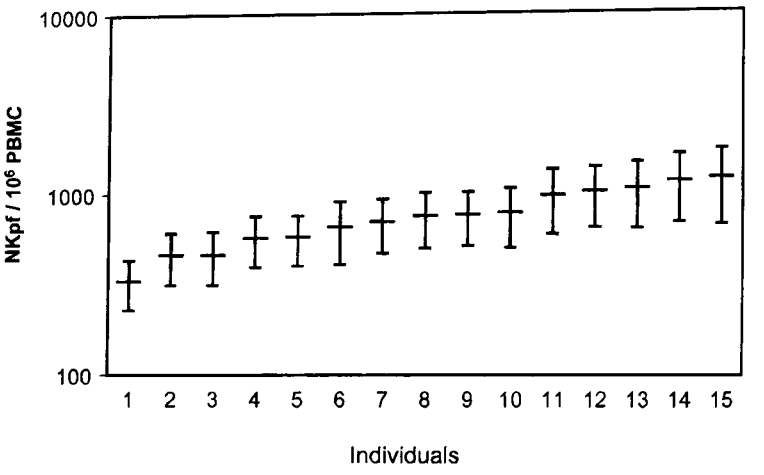

Fig. 4. Mean NKpf with 95% CI plotted on a $\log_{10}$ scale (10^2-10^4) in 15 individuals ranked from lowest to highest. PBMC were cultured with 25 Cetus unit/ml rIL-2 plus 10 ng/ml rIL-15, and cells were assayed after 5 days.

3.4. Individual variation in NKpf

We next investigated the variation between 15 individuals tested in a 5-day assay to which 25 IU/ml rIL-2 and 10 ng/ml rIL-15 were added on day 0. NKpf estimates ranged between 320 (95% CI 228–430) and 1300 (95% CI 672–1746) per 10^6 PBMC. These were ranked from the lowest to the highest and were numbered 1 to 15 (Fig. 4). Technical variation was an unlikely explanation since five repetitions of the assay with one individual gave a median NKpf of 1503 and a sampling error of $\pm$ 190 per 10^6 PBMC.

4. Discussion

In this report, we have described a novel method for quantifying the number of precursors of functionally active NK cells within a PBMC sample. We have shown that proliferation and maturation of functionally viable NK cells was absolutely dependent on the continued presence of rIL-2 and rIL-15 in the culture and that functional viability dwindled in their absence. Following allo-activation with an irradiated pool of PBMC, NKpf was lower than when cytokines were added. Individual differences in NKpf estimates between 15 adults ranged from 300 (0.03%) to 1500 (0.15%) per 10^6 PBMC. The observation that rIL-2 and rIL-15 were equally effective is consistent with the view that they both share the same signalling pathway.

The novelty of this assay lies in its ability to estimate the full potential NK function. Unlike short-term qualitative assays used extensively elsewhere, this assay permits accurate estimates of the potential NK function within a blood sample. NKpf rose to a plateau by day 7 and the increment in NKpf between days 7 and 10 was insignificant, suggesting that all available precursors had matured into functionally viable NK cells. In vivo, the story may be different since these mature NK cells will be dependent on the prevailing cytokine microenvironment and may not be sustained. The observed variation in NKpf between healthy individuals reflects variables that have yet to be fully explored, such as clone size and number.

The present assay is based on a modification of the well established non-radioactive Eu-release assay. (Blomberg et al., 1986a,b; Nagao et al., 1996). It is relatively simple, sensitive and non-hazardous. The Eu-release method has been shown to be as sensitive as the ^{51}Cr-release method for measuring functional NK cells Furthermore, NKpf estimates are relatively insensitive to target cell concentration since the presence or absence of Eu-release over and above the background plus three times the SD is considered to be a qualitative indicator of a functional NK clone(s). We confirmed this assumption by showing that NKpf estimates obtained with 5000 or 10,000 target cells/well were not significantly different (unpublished data).

Both rIL-2 and rIL-15 drove proliferation and maturation as evidenced by the plateau at day 7 of culture. When both cytokines were given together, there was no increment in NKpf, suggesting that the same signalling pathways were used to equal effect by both cytokines. However, in the absence of experiments with purified subsets, it is impossible to know whether these cytokines were operating directly through receptors on NK cells and/or indirectly via helper T cells in the culture. Since receptors for both cytokines shared the same β and common γ chain, they were likely to have used the same signalling pathway (Carson et al., 1997; Waldmann et al., 1998; Waldmann and Tagaya, 1999). Human IL-15 has been shown to use the IL-2R and its protein sub-units for binding and signal transduction in NK cells, and it has a distinct effect on the CD56+ subset. Furthermore, cord blood cells treated with rIL-15 exhibited augmented NK activity and an increased percentage of CD56+16+ cells (Mrozek et al., 1996; Gaddy and Broxmeyer, 1997; Nguyen et al., 1998).

In vivo NK clonal expansion would be subject to regulatory mechanisms leading to clonal contraction. Similar processes might operate in vitro and explain why the NKpf with allo-activation alone was less than when cytokine was added. If, in a proportion of culture wells, clonal contraction through apoptosis occurred, this would result in lower NKpf estimates. NK cells destined for apoptosis could have been 'rescued' by rIL-2 added to the allogeneic stimulus and this may explain the synergistic effect of the two stimuli. However, the increment associated with allogeneic stimulus plus rIL-2 observed as early as day 1 (see Fig. 2) supported the view that two populations of cells were activated, namely the NKp and the NK-Tp.

For the future, we intend to apply this NKpf assay to monitor the effects of surgery, blood transfusion and transplantation. We have already shown that the early signalling pathways leading to proliferation and maturation of functional NK cells are highly resistant to immunosuppressive drugs such as cyclosporin, tacrolimus (Haque et al., 1999) and rapamycin (unpublished). Furthermore, we have speculated that once armed with allo-antibody, NK cells may become vehicles of graft destruction and acute rejection.

Peptide-specific T cell precursor frequencies measured by limiting dilution analysis (LDA) are reputedly lower than frequencies estimated by tetramer analysis without LDA. This may be a consequence of the need for interactions between several different cell populations during T cell maturation (e.g. T cells and dendritic cells). Maturation and proliferation of NKp requires only the presence of soluble growth factors and may be less susceptible to this artifact. Tetramer analysis using a range of HLA Class I peptides would complement this NK functional assay by offering an insight into the distribution of inhibitory receptors, but it would be wrong to assume that all cells carrying inhibitory receptors had the potential to lyse NK targets and vice versa. Hence, the shortcomings observed with estimates of T cell precursors are unlikely to be applicable in this case. We suggest that the method described here gives more accurate estimates of the potential number of NKp than previous assays.

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